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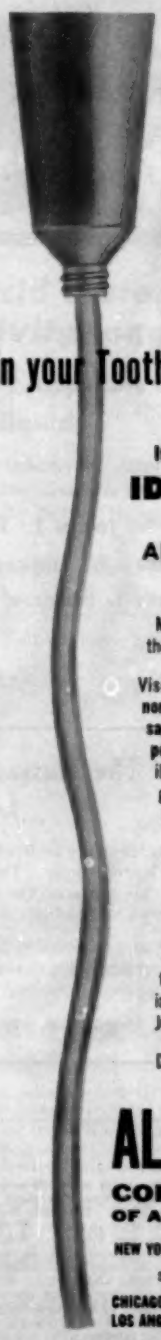

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VOLUME XLVII — *MAY 1958* — NUMBER 5

A Study of the Effects of Ultrasonics on the Particle Size of Certain Suspended Pharmaceuticals*

By OSCAR E. ARAUJO† and PATRICK F. BELCASTRO

Data are presented showing the influence of insonation intensity, time of exposure, concentration of suspended material, and addition of dispersing agents on the particle size and rate of sedimentation of certain medicaments. All suspensions exposed to ultrasonics have a smaller particle size diameter, a slower rate of sedimentation, and are more stable than the control or noninsonated suspensions.

THE EFFECTS of ultrasonics on matter can be attributed to cavitation, tremendous mechanical forces, heat, depolymerization, and other factors. The first significant study of the thermal, biological, and chemical effects of ultrasonic vibrations was reported by Wood and Loomis (1). Myers and Goodman (2) applied the mechanical forces of this energy in devising an ultrasonic homogenizer, the "Rapisonic," which produced a calamine lotion stable for three months. Recently, Misek and Skauen (3) reported certain effects of ultrasonics on pharmaceutical dispersions. Other investigators (4–7) have studied the value of ultrasound as a means of dispersing solids in a liquid medium.

This work is an attempt to evaluate quantitatively certain pharmaceutical suspensions which have been exposed to the mechanical forces of ultrasonics with the ultimate purpose of producing preparations that are superior to control or noninsonated samples. The rate of sedimentation and the particle size diameters were the parameters chosen for evaluating the suspensions mentioned in this paper.

APPARATUS

The apparatus used in this study consisted of a 250-watt radio-frequency generator with a variable frequency range of 100 to 1000 kilocycles per second (8). The Hypersonic Transducer Model BU-305 used in conjunction with the generator operates at a frequency of 400 kilocycles per second. The transducer contains a concave bowl of barium titanate which focuses the sound energy for an area of 0.5 square cm. at a point 57.4 mm. above the center of the element.

EXPERIMENTAL

The insonation chamber used in this investigation consisted of a 25 x 150 mm. lipless pyrex test tube, to which was adapted a glass water-jacket so that cold water could be continuously circulated to cool the suspension during insonation.

A volume of 20 ml. of suspension was employed throughout the study. Four insoluble medicaments commonly administered in the form of aqueous suspensions were selected: zinc oxide, bismuth subcarbonate, sulfathiazole, and procaine penicillin G. These substances are representative ingredients of external, oral, and parenteral pharmaceutical suspensions.

* Received August 12, 1957, from the Research Laboratories of the Purdue University School of Pharmacy, West Lafayette, Ind.

† Present address: School of Pharmacy, Ohio Northern University, Ada.

Effect of Ultrasonics on Dilute Suspensions.—A spectrophotometric method was developed to evaluate the relative effect of ultrasonic energy on the rate of sedimentation of the previously mentioned medicaments. The suspensions were simple systems of the substance in distilled water. They were exposed to ultrasonics for periods of either 5, 10, or 15 minutes. Ultrasonic intensities at rheostat settings of 30, 70, and 110 were employed, representing energies estimated at 28.5, 119, and 365 watts, respectively. However, since ultrasound concentrates the sound waves, the actual energy at the focal point may far exceed the above values.

The Beckman Model B spectrophotometer was employed to determine the absorbance of the suspensions. All experiments were carried out at a wavelength of 500 mμ. Absorbance can be directly related to the rate of sedimentation of solid particles in suspension, this rate being a function of the particle size of the solid material. As the particles settle out of the light path, the absorbance decreases at a rate inversely proportional to the size of the particles in suspension.

Since it was impractical to extend the experimentation period to a point where all the particles were no longer in the light path, all absorbance values were measured at intervals of time, only until the initial absorbance was reduced to 50%. In this manner, a partial rate of sedimentation was determined which excluded only the very small particles.

The insonated suspensions were compared to control suspensions to observe differences in rate of settling due to the effect of ultrasonic vibrations. The concentration of the solid used is restricted by the quanta of light that can be transmitted through the suspension, to give a reading on the spectrophotometer, thus limiting this phase of the work to the use of dilute suspensions.

Table I shows the rate of sedimentation of the suspensions studied.

The results clearly indicated the effect of increasing intensity of ultrasonics. In the case of all four substances, a progressively greater reduction in particle size, as evidenced by retardation of the rate of sedimentation, occurred with a corresponding increase in ultrasonic intensity.

By the same token, as the time of exposure to ultrasound was prolonged, the rate of sedimentation of the solid particles in all suspensions was correspondingly retarded.

These facts would lead to the conclusion that the longer the period of insonation and the higher the intensity of ultrasound, the more pronounced is the reducing effect on the particle size of the suspended solids.

However, the data from the tables show that the extent of the effect of ultrasonics varied with each compound. A comparison between the time required for a control suspension of zinc oxide to reach the "half-life" point (50% decrease in absorbance), and the time required for the suspension insonated at maximum conditions of exposure and intensity to reach the corresponding stage, showed that the rate of sedimentation ratio was 1 to 4.9.

Since, according to Stokes' law, the rate of sedimentation varies directly with the square of the diameter of the particle, it seemed logical to hypothesize that the particle size of the solid in the control suspension was 2.2 times as large as that in the suspension insonated at maximum conditions of exposure and intensity.

TABLE I.—EFFECT OF ULTRASONICS ON THE SEDIMENTATION RATE OF VARIOUS MEDICAMENTS

% Decrease in Absorbance	Non-insonated	Intensity of 30 for 5 min.	Intensity of 30 for 10 min.	Intensity of 30 for 15 min.	Intensity of 70 for 5 min.	Intensity of 70 for 10 min.	Intensity of 70 for 15 min.	Intensity of 110 for 5 min.	Intensity of 110 for 10 min.	Intensity of 110 for 15 min.
	Minutes Elapsed									
	Zinc Oxide Suspension 0.03%									
5	2.9	5.0	6.1	6.5	14.1	19.8	30.2	21.2	23.2	33.1
20	9.1	19.1	23.8	27.3	32.3	35.4	46.2	40.4	39.3	62.4
30	13.2	25.4	31.6	35.2	41.8	45.2	57.5	50.7	51.2	76.1
50	22.4	38.8	47.5	53.8	64.9	71.9	87.4	79.1	88.9	108.7
	Bismuth Subcarbonate Suspension 0.03%									
	0.7	1.5	1.6	1.9	3.1	2.7	3.6	4.2	4.1	4.9
20	3.1	6.5	7.0	9.1	13.0	10.6	14.4	20.6	27.3	41.5
30	5.6	11.5	16.4	16.8	23.0	20.0	30.8	36.2	52.4	98.0
50	14.8	46.6	58.4	62.4	74.3	78.1	101.4	121.7	...[a]	...[a]
	Sulfathiazole Suspension 0.5%									
5	0.2	1.3	1.5	1.4	1.6	1.4	1.4	1.8	2.9	1.8
20	1.1	4.4	5.6	6.3	8.2	6.4	8.1	9.5	13.4	11.7
30	1.8	6.7	8.3	9.5	13.1	11.4	14.7	17.8	22.0	21.0
50	4.3	12.3	14.8	18.5	24.4	27.3	32.8	42.1	48.1	50.0
	[b] Penicillin Procaine G Suspension 1.0%									
5	0.5	0.6	0.7	0.6	0.6	0.7	0.8	0.7	1.1	1.2
20	1.8	2.2	2.2	2.4	2.2	3.0	3.0	2.8	4.2	5.0
30	2.8	3.5	3.5	4.2	3.9	4.8	5.5	5.4	7.5	9.6
50	5.7	8.9	10.5	11.5	12.2	13.5	15.8	19.2	20.9	26.3

[a] Measurements not taken due to indefinitely long periods of sedimentation.
[b] Obtained through the courtesy of Chas. Pfizer and Co., Inc., Brooklyn 6, N. Y.

A similar comparison of the bismuth subcarbonate suspensions was impossible, since the suspension insonated at maximum conditions never reached its "half-life" point during the course of these experiments. However, at the 30% point in the decrease of the absorbance, the rate of sedimentation ratio observed was 1 to 17.5. This indicated, following the previously mentioned reasoning, that the particle size of the medicament in the control suspension was 4.2 times larger than that of the insonated suspension.

The sulfathiazole suspensions thus evaluated showed a sedimentation ratio of 1 to 11.6, and the particle size of the solid in the control suspension was 3.4 times larger than that of the insonated suspension.

The procaine penicillin G suspensions exhibited a sedimentation ratio of 1 to 4.6, and the particle size of the suspended medicament in the control suspension was 2.1 times greater than that in the insonated suspension.

From these data, it can be seen that the maximum effect of ultrasonics was observed with the bismuth subcarbonate suspensions, followed in order by sulfathiazole, zinc oxide, and procaine penicillin G.

It must be noted, however, that at maximum conditions of insonation both sulfathiazole and procaine penicillin G suspensions showed some evidence of decomposition, exhibiting a change in color and developing a disagreeable odor.

Effect of Ultrasonics on Concentrated Suspensions.—At this point in the study, it was desired to evaluate the effect of ultrasound on more concentrated suspensions of the previously mentioned compounds. Since it was evident from the foregoing results that the greatest effect was evidenced when the suspensions were subjected to insonation at an intensity of 110 (estimated at 365 watts) for a period of fifteen minutes, these conditions were used exclusively in this phase of the investigation. Since sulfathiazole and procaine penicillin G previously showed evidence of decomposition, further investigation of these suspensions was abandoned.

The spectrophotometric method previously used to evaluate the suspensions not only was impractical for high concentrations, but provided only relative particle size relationships by means of an indirect parameter, such as absorbance. Thus, it became necessary to find a method that would provide an entire particle size distribution of the solids in suspension and that would also furnish data for calculation of absolute mean diameters of the particles. A liquid elutriation method was investigated, but it proved to be insufficiently sensitive to classify particles smaller than 10 microns. Finally the Andreasen pipet, as described by Schweyer (9) produced satisfactory results. This apparatus operates on the principle that if samples are taken from a constant level near the bottom of a cylinder containing a suspension, at appropriate intervals of time, Stokes' law can be applied and the particle size distribution of the suspension can be calculated.

Since increasing the concentration of any solid material in a liquid medium results in a greater tendency towards particle flocculation in the suspension, a small amount of a protective colloid and a dispersing agent was added. Three different media were therefore used: distilled water, distilled water and 0.1% sodium carboxymethylcellulose (CMC), and distilled water and 0.1% Marasperse C. In the case of bismuth subcarbonate, a combination of 0.05% CMC and 0.05% Marasperse C in distilled water was used in lieu of 0.1% Marasperse C, because better initial dispersion was thus obtained. Three concentrations of the solid materials were used: 2, 5, and 10%.

Figures 1, 2, and 3 show sample plots of the particle size distribution obtained from the data. Tables II and III indicate the relative effect of

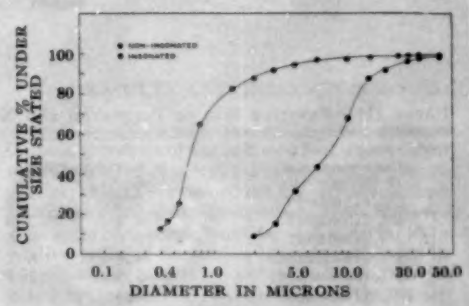

Fig. 1.—Particle size distribution of 2% zinc oxide suspensions in distilled water.

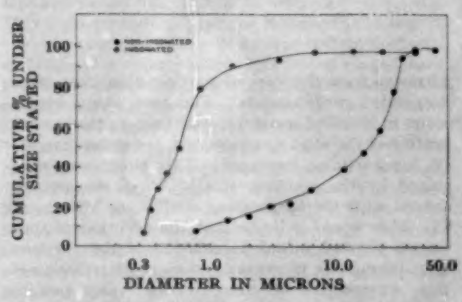

Fig. 2.—Particle size distribution of 2% zinc oxide suspensions containing 0.1% CMC.

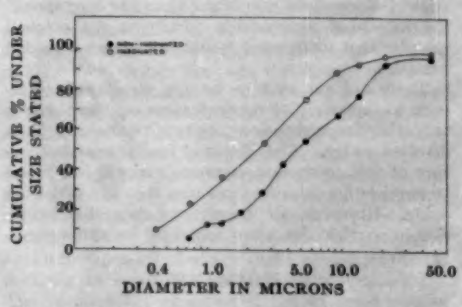

Fig. 3.—Particle size distribution of 2% bismuth subcarbonate suspensions in distilled water.

TABLE II.—PARTICLE SIZE OF INSONATED AND NONINSONATED ZINC OXIDE SUSPENSIONS

Concentration of Zinc Oxide	ZnO in Distilled H_2O		ZnO with 0.1% CMC		ZnO in 0.1% Marasperse C[c]	
	$d_{av.}$[a]	s[b]	$d_{av.}$	s	$d_{av.}$	s
2% (Control)	8.5	6.5	14.5	9.6	1.8	3.5
5% (Control)	22.1	18.5	19.8	10.9	1.2	2.2
10% (Control)	26.9	8.2	17.3	11.0	1.5	3.4
2% (Insonated)	1.2	1.6	0.85	0.94	0.58	0.24
5% (Insonated)	1.9	3.5	9.6	9.6	0.64	0.36
10% (Insonated)	26.9	9.9	12.9	11.0	0.71	0.50

[a] Arithmetic mean diameter in microns.

[b] Standard deviation calculated from the formula: $s = \sqrt{\frac{\Sigma nd^2}{\Sigma n} - d_{av.}^2}$

[c] Marathon Corporation, Rothschild, Wis.

TABLE III.—PARTICLE SIZE OF INSONATED AND NONINSONATED BISMUTH SUBCARBONATE SUSPENSIONS

Concentration of Bi Subcarbonate	In Distilled Water		With 0.1% CMC		With 0.05% CMC and Marasperse C	
	$d_{av.}$[a]	s[b]	$d_{av.}$	s	$d_{av.}$	s
2% (Control)	7.3	7.2	4.9	4.7	5.3	4.9
5% (Control)	8.1	7.8	5.5	8.2	5.2	6.0
10% (Control)	9.0	8.6	4.8	5.0	5.2	5.1
2% (Insonated)	4.0	5.1	3.4	3.3	3.8	4.5
5% (Insonated)	3.8	5.4	4.5	4.4	3.4	2.8
10% (Insonated)	4.5	4.2	4.0	3.6	4.5	4.6

[a] Arithmetic mean diameter in microns.

[b] Standard deviation calculated from the formula: $s = \sqrt{\frac{\Sigma nd^2}{\Sigma n} - d_{av.}^2}$

ultrasonics on the various particle diameters of the suspended medicaments. The zinc oxide suspensions in distilled water showed that as the concentration of the solid was increased, the mean diameter of the particles increased. This effect was minimized by the addition of CMC and was not observed with the suspensions containing Marasperse C. This would indicate that the addition of these agents prevent initial flocculation of the particles, thus permitting ultrasonics to exert its reducing effect, irrespective of concentration. One possible explanation is that by keeping the particles in a deflocculated form, these agents permit the ultrasonic waves to reach the individual particles more readily and thereby exert their reducing effect. However, in all cases, the mean particle size of the solids in the control suspensions was larger than the corresponding insonated suspensions, justifying the original premise that ultrasound would break up suspended particles.

There did not seem to be any significant difference in the effect of concentration on the bismuth subcarbonate suspensions, even with those in plain distilled water. The chemical and physical structure of this compound seems to make it less prone to particle flocculation than was the case with zinc oxide. However, all suspensions showed a reduction in particle size when subjected to ultrasonics.

SUMMARY

1. The effect of ultrasonic intensity and time of insonation on dilute suspensions of four commonly used medicaments was studied.

2. The effect of ultrasonic vibrations on more concentrated suspensions of zinc oxide and bismuth subcarbonate was reported.

3. The influence of the addition of a suspending agent (CMC) and a deflocculating agent (Marasperse C) to suspensions of zinc oxide and bismuth subcarbonate was observed.

4. In all cases, ultrasonic energy reduces the particle size of the suspended medicament and retards its rate of sedimentation, providing a more stable preparation.

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Anti-inflammatory Effects of Romilar CF*

By LOWELL O. RANDALL and JOSEPH J. SELITTO

Analgesic, antiedematous, and antipyretic effects of Romilar, N-acetyl-*p*-aminophenol, *l*-phenylephrine, chlorpheniramine and a mixture of these ingredients have been demonstrated on the inflamed foot of the rat. Codeine has primarily a central analgesic effect; salicylate and acetophenetidin have primarily a peripheral analgesic, antipyretic, and antiedematous effect while Romilar, N-acetyl-*p*-aminophenol, phenylephrine, and chlorpheniramine and a mixture of these ingredients have mixed central and peripheral effects.

ROMILAR® (DEXTROMETHORPHAN) is a cough suppressant which has the clinical antitussive effectiveness of codeine (1). Romilar® does not have the potent analgesic effects and addictive properties of narcotics (2–4). In order to provide stronger analgesic properties and more decongestant properties, a mixture of Romilar®, chlorpheniramine, *l*-phenylephrine and N-acetyl-*p*-aminopbenol has been prepared. The analgesic-antipyretic effectiveness of N-acetyl-*p*-aminophenol was discovered by Flinn and Brodie (5) and confirmed by Boreus and Sandberg (6) and Wallenstein and Houde (7). This paper is concerned with the comparison of the analgesic, antipyretic, and antiedematous effects of the various ingredients alone and the complementary effectiveness of the ingredierts in a mixture.

EXPERIMENTAL

Inflammation in the rat's foot was induced by the injection of 0.1 ml. of a 20% suspension of brewer's yeast into the plantar surface. Thresholds to pain in the inflamed and control feet were measured by the pressure method of Randall and Selitto (8). The circumferences of the control and inflamed feet were measured with a loop of string attached to a millimeter scale according to the procedure of Selitto and Randall (9). Temperatures of the feet and the skin of the back were measured with the Universal Thermometer manufactured by Electro Laboratory of Copenhagen.

Drugs were injected subcutaneously into five rats at each dose level and five controls were used. The changes in pain threshold, size, and temperature of both inflamed and control feet, and of skin temperature were measured at intervals of 1, 2, and 4 hours after the administration of the drug. The degree of analgesia was calculated as the increase in mm. Hg in the pain threshold of treated animals in comparison with controls. Temperature changes of the feet and skin were recorded as the difference in degrees centigrade between treated and control animals. Antiedematous effects were calculated as the per cent inhibition of the swelling of the feet of treated animals in comparison with the control animals.

* Received August 20, 1957, from the Department of Pharmacology, Hoffmann-La Roche, Inc., Nutley 10, N. J.

RESULTS AND DISCUSSION

Analgesic effects as indicated by the increase in the pain threshold of the inflamed foot were evident with Romilar®, N-acetyl-*p*-aminophenol, *l*-phenylephrine, chlorpheniramine, and the mixture of these ingredients. Also analgesic effects were observed with salicylate, acetophenetidin and codeine. The relative order of decreasing potency calculated as the dose in mg./Kg. which induced an increase in threshold of 120 mm. Hg was as follows: chlorpheniramine 1, *l*-phenylephrine 8, codeine 10, Romilar® 10, salicylate 100, acetophenetidin 200, and N-acetyl-*p*-aminophenol 400.

The Romilar® CF induced an increase in the pain threshold also. A dose of 2 cc./Kg. orally of the mixture raised the threshold over 100 mm. Hg. This dose contained 6 mg. of Romilar®, 0.5 mg. of chlorpheniramine, 2 mg. of *l*-phenylephrine, 48 mg. of N-acetyl-*p*-aminophenol. As tested on inflamed tissue this combination had an antipyretic-analgesic effectiveness approximately equal to the narcotic analgesic effects of 10 mg. of codeine.

Significant analgesic effects as measured on the pain threshold of normal noninflamed foot was observed only with relatively high doses of codeine, Romilar®, N-acetyl-*p*-aminophenol, *l*-phenylephrine, chlorpheniramine, and the mixture. These effects appeared only at doses 2 to 4 times those which were effective on the pain threshold of the sensitive inflamed foot.

Coincident with the elevation of pain threshold of the inflamed foot, there is reduction of swelling and temperature of the foot with all compounds except codeine. These results are interpreted as indicating that only codeine has true central analgesic effects and lacks any peripheral antipyretic and antiedematous effects. In contrast, acetophenetidin and salicylate have primarily a peripheral action in that they elevate the pain threshold and reduce edema and temperature of the inflamed foot without affecting the pain threshold of the normal foot. The mixed effects of Romilar®, N-acetyl-*p*-aminophenol, *l*-phenylephrine, chlorpheniramine, and the Romilar® CF mixture in elevating pain thresholds, reducing edema and lowering temperature indicates a mixed central and peripheral effect of these compounds. There is the possibility that the peripheral anti-inflammatory effects of these compounds could partially be explained on a circulatory basis also. The permeability of inflamed tissues could readily be changed either by an increase or a decrease in circulation rates and pressures. Under the experi-

TABLE I.—ANTI-INFLAMMATORY EFFECTS IN RATS

Compound	Dose, mg./Kg.	Route	Time Hr.	Analgesia: Infl. Foot mm. Hg Increase	Analgesia: Normal Foot mm. Hg Increase	Anti-edema % Inhibition	Antipyresis: Infl. Foot °C. Change	Antipyresis: Normal Foot °C. Change	Antipyresis: Skin °C. Change
Romilar®	6.25	P.O.	1	58	0	0	−1.6	−0.2	−0.1
			4	10	0	0	0	0	0
Romilar®	12.5	P.O.	1	130	26	13	−1.9	−0.2	−0.3
			4	42	0	0	−0.6	−0.1	0
Romilar®	25.0	P.O.	1	232	123	23	−2.3	+0.4	+0.1
			4	210	95	20	−0.6	0	0
N-acetyl-*p*-aminophenol	100.0	P.O.	1	4	0	0	0	0	0
			4	0	0	0	0	0	0
N-acetyl-*p*-aminophenol	200.0	P.O.	1	100	32	14	−1.6	0	−0.4
			4	0	0	0	0	0	0
N-acetyl-*p*-aminophenol	400.0	P.O.	1	235	123	44	−3.9	−1.7	−2.0
			4	220	115	30	−1.4	−0.9	−1.6
Acetophenetidin	200.0	P.O.	1	109	36	18	−1.3	−0.2	−1.0
			4	0	20	0	+0.1	+0.7	−0.4
l-phenylephrine	2.5	P.O.	1	8	0	0	+0.2	+0.1	+0.4
			4	0	0	0	0	0	0
l-phenylephrine	5.0	P.O.	1	71	23	0	−0.6	−0.7	−0.4
			4	7	0	0	0	0	0
l-phenylephrine	10.0	P.O.	1	129	42	0	−0.3	−0.5	−0.1
			4	24	7	0	0	0	0
Chlorpheniramine	2.5	P.O.	1	69	12	9	−0.8	−0.5	−0.2
			4	0	0	0	0	0	0
Chlorpheniramine	5.0	P.O.	1	124	49	12	−0.9	−0.9	−0.4
			4	0	0	0	0	0	−0.4
Chlorpheniramine	10.0	P.O.	1	204	122	19	−1.2	−1.2	−0.6
			4	23	7	0	0	0	0
Romilar® CF, cc./Kg.	0.25	P.O.	1	44	4	9	+0.2	+0.1	0
			4	0	0	0	0	0	0
1 cc. contains:									
3 mg. Romilar®	0.5	P.O.	1	69	32	14	−0.2	−0.1	+0.9
			4	0	0	0	0	0	0
0.25 mg. chlorpheniramine									
1 mg. *l*-phenylephrine	1.0	P.O.	1	80	24	19	−0.4	+0.3	−1.0
			4	0	0	0	0	0	0
24 mg. N-acetyl-*p*-aminophenol	2.0	P.O.	1	114	48	19	−1.6	−0.1	−0.8
			4	7	0	0	0	0	0
	4.0	P.O.	1	176	84	29	−1.3	−0.2	−0.5
			4	41	0	0	−0.6	0	0
	8.0	P.O.	1	240	97	29	−2.6	−0.6	−1.3
			4	74	58	0	−0.9	+0.7	+0.2
Codeine	6.25	P.O.	1	58	35	0	0	0	0
			4	0	0	0	0	0	0
Codeine	12.5	P.O.	1	144	92	0	0	0	0
			4	12	0	0	0	0	0
Codeine	25.0	P.O.	1	235	136	0	0	0	0
			4	103	38	0	0	0	0
Na Salicylate	50.0	P.O.	1	40	0	14	+0.3	+0.7	+0.1
			4	0	0	0	0	0	0
Na Salicylate	100.0	P.O.	1	116	0	19	−1.5	0	0
			4	45	0	0	0	0	0
Na Salicylate	200.0	P.O.	1	209	0	24	−2.6	−0.2	+0.1
			4	132	0	10	−1.0	0	0

mental conditions employed in producing the edema in the rat's foot and relieving it with various agents, circulatory effects could exert important changes which might be measured as apparent antipyretic, antiedematous, and analgesic effects. This question has not been adequately resolved.

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Paper Chromatography of I^{131}-Labeled Iodopropylidene Glycerol With Autoradiographic Identification*

By GEORGE F. HOFFNAGLE†, CATHERINE N. SIDERI, and ARTHUR OSOL

The paper chromatographic characteristics of iodopropylidene glycerol in various solvent systems were investigated. Certain quantitative evaluations of the chromatographic data were made possible through tagging of the compound with I^{131}. Autoradiograms of chromatograms were also prepared, in connection with which the relationship of radioactivity of test specimens to duration of exposure in preparing the autoradiograms was studied.

THIS STUDY WAS OCCASIONED by the need for developing suitable analytical techniques to be used in certain phases of an investigation of the biological distribution and fate of iodopropylidene glycerol (1, 2), hereafter referred to as IPG, a safe and orally efficacious source[1] of iodine for nutritional and therapeutic use in humans (3–6).

Specifically, knowledge of the chromatographic characteristics of IPG in various solvent systems was required in order to permit identification of the I^{131}-labeled compound(s) in the microgram quantities present in preparations of biological material (1). Location of the "spots" obtained by paper chromatography of tagged IPG was effected by scanning the paper with a Geiger-Mueller tube and attached counter. More exact delineation of the spots was achieved by preparing on X-ray film autoradiograms of the chromatograms.

EXPERIMENTAL

Chromatographic Studies.—An ascending technique, along lines described by Williams and Kirby (7), was chosen for this work. The IPG, tagged with I^{131} by an exchange method (2), was applied to the paper as a solution in chloroform. The amount of IPG applied to the paper was determined from counts of total radioactivity obtained with the scanning equipment before the chromatography was begun. Sheets of Whatman No. 1 paper were used. Applications of the solution ware made at points 5 cm. apart along a line parallel to the bottom edge of the paper and 1 cm. above the solvent level. The paper was made into a cylinder by securing the vertical edges with paper clips. The chromatography chamber was saturated with the vapor of the solvent through the night preceding the start of each chromatographic experiment.

The following solvent systems were investigated: (*1*) The upper phase of an equilibrium mixture of *n*-butanol and 2 *N* formic acid in water. (*2*) The upper phase of an equilibrium mixture of 20% *p*-dioxane in *n*-butanol with a 2 *N* aqueous solution of ammonia. (*3*) The upper phase of an equilibrium mixture of *n*-butanol with 0.2 *M* sodium phosphate buffer at pH 7.2. (*4*) Isopropyl ether saturated with water. (*5*) Toluene saturated with water. (*6*) The ethyl acetate phase of a 5:1 equilibrium mixture of ethyl acetate and water. (*7*) The lower phase of an equilibrium mixture of 11 volumes of carbon tetrachloride, 2 volumes of *p*-dioxane, and 1 volume of water. (*8*) The upper phase of an equilibrium mixture of 10 volumes of toluene, 5 volumes of isopropyl ether, and 1 volume of water. (*9*) Water alone. (*10*) Water saturated with *n*-butanol. (*11*) A 2-phase system composed of an immobile phase of 2% glacial acetic acid in propylene glycol, and a mobile phase of a 1:1 mixture of benzene and cyclohexane. (*12*) A 2-phase system composed of formamide (applied as a 30% solution in acetone) as the immobile phase, with a mixture of 20 volumes of isooctane, 10 volumes of benzene, and 1 volume of formamide as the mobile phase.

The butanol-phosphate buffer system, represented by (*3*) above, was chosen for the biological studies because the IPG isomers did not appreciably separate, but were sufficiently well separated from other expected iodine compounds, and the pH of the buffer prevented decomposition of iodides to free iodine.

Detection and Quantitative Evaluation of Radioactive Spots.—The completed chromatograms were air-dried, then scanned for location of radioactive spots using an end-window, organic-vapor-quenched, Geiger-Mueller counter tube enclosed in a 1.25 cm. lead shield, inverted under a 50 × 50 cm. Plexiglas table of 0.65 cm. thickness (Fig. 1). A 2 × 2 cm. opening in the table and in the lead shield over the window of the counter tube permitted counting over an area of 4 sq. cm.; light entering through the Plexiglas outlined the area of the paper being counted

By ruling off at right angles distances of 2 cm. from the point of application of the material which had been chromatographed, the path of the migrating materials could be accurately surveyed. The initial counts of radioactivity were for one minute. The rulings permitted easy and reproducible return to any area of the paper to obtain replicate and

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This investigation was supported in part by a grant from Henry K. Wampole & Co., Inc., Philadelphia, Pa.
† Present address: 86 Forest Ave., Verona, N. J.
[1] Preparations of IPG used in this study were supplied by Henry K. Wampole & Co., Inc., Philadelphia, Pa.

Fig. 1.—Scanning table for detection and estimation of radioactive substances on paper chromatograms.

longer counts. An estimate of relative concentrations of radioactivity of different areas on a chromatogram was obtained by subtracting the background (usually about 30 counts per minute) from the counting rate for each area, then dividing the value for each area by the sum of the values for all areas.

Autoradiographic Studies.—To facilitate location and delineation of radioactive spots, autoradiograms of the chromatograms were prepared. The film used was Kodak type K X-ray film, in 1 7/8 × 16 inch strips. Boyd (8) found that for low intensity exposures this film is more sensitive to I^{131} than No-Screen and other commonly available X-ray films. The film was placed flat directly over the chromatogram along the line of travel of the sample and stapled on. The origin and solvent front lines were marked on the film with India ink at the time of placement.

It was desired to continue the autoradiographic exposure long enough to record weakly radioactive spots, which procedure resulted in overexposure of strongly radioactive spots. In this connection, it was considered important to correlate the observed count of a sample on the scanning table with exposure time of the film. Pelc (9) recommended an exposure time corresponding to 10^7 disintegrations per sq. cm. A series of dilutions of a sodium radio-iodide solution standardized by Abbott Laboratories, diluted in 1% potassium iodide solution, was prepared and the corrected counts per minute obtained on the scanning table. By this method a ratio of 283 ± 4.8 counts per minute per millimicrocurie was established over the range of 36 to 1125 counts per minute, or 0.13 to 4 millimicrocuries.

A series of identical radioactive spots was prepared on Whatman No. 1 paper, having a corrected count of 275 counts per minute (0.97 millimicrocurie). After drying, a square of Kodak type K X-ray film was placed directly over each spot. The films were removed at various times as indicated in Fig. 2. All the squares were developed at the same time, in strict accordance with directions for the film. It will be noted that at 10^7 disintegrations, approximately maximum density of the image was obtained, and adequate density for detection was obtained with as little as 2.5×10^6 disintegrations.

RESULTS AND DISCUSSION

The chromatographic data obtained for IPG in different solvent systems, expressed in terms of the

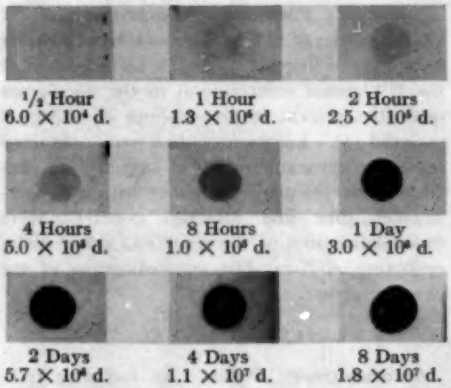
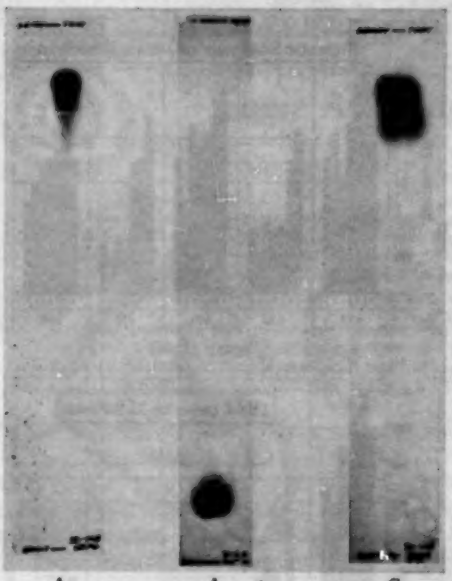


Fig. 2.—Effect of varying exposure time to identical I^{131} spots, in terms of reaction density of Kodak Type K film.

TABLE I.—CHROMATOGRAPHIC DATA FOR IPG IN VARIOUS SOLVENT SYSTEMS

Solvent	Spot 1		Spot 2		Spot 3	
	R_f Value	Component, %	R_f Value	Component, %	R_f Value	Component, %
1	0.79	6	0.85	39	0.89	55
2	0.61	92[a]	0.90	5	0.93	3
3	0.80	7	0.84	32	0.89	61
4	0.66	6	0.88	50[a]	0.93	44
5	0.00	79	0.97	21[a]	..	..
6	0.96	100[a]	..	..	..	..
7	0.00	16	0.91	24	0.97	60
8	0.96	100[a]	..	..	..	..
9	0.82	100[a]	..	..	..	..
10	0.79	55	0.84	45	..	..
11	0.03	42	0.89	58[a]	..	..
12	0.00	75	0.75	25[a]	..	..

[a] Noticeable tail on autoradiogram and scanning.



A. A. C.

Fig. 3.—Autoradiograms of chromatograms using solvent (3), *n*-butanol saturated with sodium phosphate buffer at pH 7.2. A. 21PG. B. Potassium iodide tagged with radioactive sodium iodide. C. Mixed IPG.

R_f value for the spot or spots observed, with an estimate of the proportion of the component producing each spot, are given in Table I. Examples of autoradiograms of chromatograms obtained with solvent No. 3 are shown in Fig. 3.

Duplicate chromatograms were run using each solvent, and in those cases where more than one spot was obtained another pair of duplicates were obtained. The appearance of multiple spots is not surprising in view of the fact that two structural isomers of IPG, differing in the position of the iodine atom in the molecule, are present, and that a theoretical total of 12 optical stereoisomers may be present, which latter, however, have not been characterized (2).

The R_f values obtained with the various solvents indicate relatively good solubility of IPG in polar solvents, including chlorinated solvents as well as water, and the relatively poor solubility of a large proportion of the preparation in hydrocarbons.

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Development of a Germicidal Soap Containing Bithionol*

By S. H. HOPPER† and K. M. WOOD‡

The use of one per cent Tween 80 in culture media reduces the inhibitory action of hexachlorophene in surgical scrub soap tests and indicates that hexachlorophene provides no appreciable reduction in bacterial count. A mixture of surface-active agents containing three per cent bithionol gave a marked reduction in bacterial count when used under identical conditions.

PREVIOUS REPORTS have described work from our laboratory on the development of a relatively inexpensive surgical scrub soap (1). The germicide in which we were interested was bithionol[1] in the amount of three per cent by volume. Using a coconut fatty acid base the reduction in bacterial count was of the same order of magnitude as a commerical preparation containing hexachlorophene. However, due to the relatively high number of personnel who developed a dermatitis on continued use of this product, it was discontinued and a search was made for a basic material which would not cause any skin reactions. This report describes the action of bithionol in a mixture of surface-active agents known as LD-44.[2] Little or no germicidal action would be expected of this anionic mixture in the absence of a specific germicidal agent.

EXPERIMENTAL

Methods.—The procedure was the serial basin

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[1] Monsanto Chemical Co., brand name Actamer for 2,2′-thiobis (4,6-dichlorophenol).
[2] Obtained from Stepan Chemical Company, Chicago, Ill.

technique as reported above for the coconut fatty acid soap. However, the culture media in this case contained 1% Tween 80[3] as the inactivating agent for the germicide. Lawrence and Erlandson, (2, 3) had shown that the inhibiting action of hexachlorophene and bithionol was nullified by Tween 80 and that plate counts made with this nonionic surface-active agent in the culture media had a considerably higher number of surviving organisms than those in which serum was used as the inactivating agent.

Patch Testing.—The prophetic patch test was done according to the description given by Schwartz (4). One hundred student nurses volunteered for this purpose. The LD-44 containing bithionol did not cause any reaction, but the adhesive tape caused quite a bit. In a group of 100, the rate of positive reactions should not exceed approximately 3% according to the statistical analysis of Henderson and Riley (5). Thus the absence of any reactions in the group tested exceeds the allowable limits quoted by these authors.

Manufacture of the Germicidal Detergent.—This is given in outline as follows.

1. Materials required: liquid detergent-44 (LD-44) obtained from Stepan, sodium hydroxide pellets obtained from Mallinckrodt, bithionol obtained from Monsanto, and perfume oil compound No. 11420 obtained from Fritzche Brothers.

2. Equipment required: mixing tanks, stainless steel, 30-gallon capacity. Agitator with variable speed, i. e., Lightening Mixer, model CV-4.

3. Formula (10 gallons): 1140.0 Gm. Actamer, 378.0 Gm. sodium hydroxide, 19000.0 cc. LD-44, 50.0 cc. perfume oil No. 11420, and distilled water to make 38000.0 cc.

4. Procedure: (a) Dissolve the sodium hydroxide in one gallon of distilled water in the pyrex bottle, then slowly add the Actamer to the hydroxide solution with constant agitation until all of the Actamer is dissolved. (b) Place the LD-44 in the stainless steel mixing tank and add the Actamer solution; mix thoroughly at low speed to avoid excessive foam formation. (c) Add the perfume and mix well, then add the remainder of the distilled water. (d) Bottle in amber containers.

RESULTS AND DISCUSSION

It is apparent from Fig. 1 that the preoperative scrub preparation containing 3% hexachlorophene did not reduce the bacterial count to any appreciable extent after eight days of continuous use. Fig. 2 shows a marked reduction in bacterial count after the LD-44 containing 3 per cent Bithionol had been used for eight days. Note that although neither preparation reduced the bacterial count to zero, the bithionol germicide reduced the bacterial flora far more effectively than the presently highly publicized hexachlorophene products. Thus the number of potentially dangerous bacteria left on the skin is also reduced. It is obvious that experimental design is tremendously important with regard to the type of results obtained; and it is therefore imperative to re-evaluate hexachlorophene in the light of the action of Tween 80 as an inhibitory agent in culture media.

It is probably true that it is not possible to sterilize the skin without destroying it. As Price (6) says, "as far as I can determine, single periods of

[3] Atlas Powder Company, Wilmington, Delaware, brand of polyoxyalkylene derivative of sorbitan monooleate.

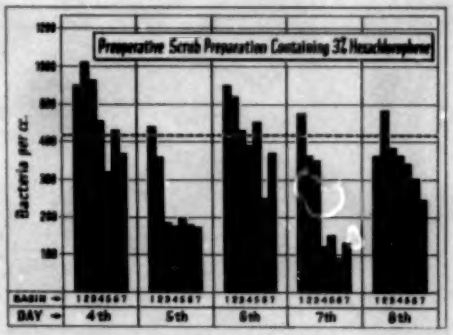

Figure 1.

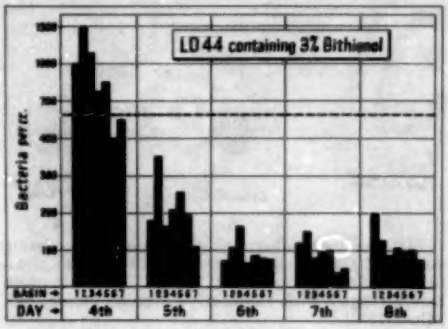

Figure 2.

washing or scrubbing from one to ten minutes with preparations containing G-11 in bar soap, liquid soap or phisoderm do not immediately reduce the cutaneous bacterial flora any more rapidly than if the washing had been done with Ivory soap."

From these observations it may be seen that a nontoxic, effective, and inexpensive germicidal scrub soap has been developed.

SUMMARY

One per cent Tween 80 in culture media reduces the inhibitory action of hexachlorophene in a surgical scrub soap and shows that there is no appreciable reduction in cutaneous bacterial count after eight days of continuous use. A mixture of surface-active agents known as LD-44 to which three per cent bithionol was added was tested under the same conditions. It did not cause any dermatitis, gave a marked reduction in bacterial count after eight days of continuous use, and reduced the bacterial flora on the hands to a considerably lower level than that obtained with the hexachlorophene preparation.

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The Synthesis of Some Substituted Thianaphthene-2-carboxamides and Their Antifungal Properties*

By ROBERT W. GOETTSCH† and GAIL A. WIESE

Thianaphthene-2-carboxylic acid, thianaphthene-2-carboxamide and nineteen substituted thianaphthene-2-carboxamides were prepared. All of the compounds were screened for their antifungal activity against *Trichophyton rubrum* and those compounds which appeared to be equal to or more active than undecylenic acid were tested against three other pathogenic fungi. The *in vitro* activity of the compounds was compared to the activity of three known antifungal agents: undecylenic acid, salicylanilide, and 5-chloro-8-hydroxyquinoline. Of all of the compounds tested, N-(2-methylpiperidyl)-thianaphthene-2-carboxamide, N-cyclohexylthianaphthene-2-carboxamide, and N-morpholinylthianaphthene-2-carboxamide were the most active against all four of the fungi.

THE PREPARATION of 3-substituted thianaphthene derivatives has undergone extensive study but only limited research has been directed toward the synthesis of the 2-substituted derivatives. This apparent lack of active research in the preparation of the 2-substituted derivatives of thianaphthene has been due to the lack of known procedures, other than metalation reactions, by which thianaphthene can be directly substituted on the 2-position.

In this investigation, thianaphthene-2-carboxylic acid, thianaphthene-2-carboxamide and nineteen substituted thianaphthene-2-carboxamides were prepared and tested for their antifungal activity. Thianaphthene-2-carboxylic acid has been prepared by several investigators by a metalation reaction followed by carbonation and acidification. Weissgerber and Kruber (1) found that the sodio derivatives prepared by the action of sodamide on thianaphthene in hot xylene yielded a mixture of thianaphthene-2-carboxylic acid and the 2,3-dicarboxylic acid. These investigators also reported the synthesis of the 2-carboxylic acid by the reaction of thianaphthene with sodamide in liquid ammonia. Schönberg, Petersen, and Kaltschnitt (2) reported the synthesis of thianaphthene-2-carboxylic acid by reacting thianaphthene with powdered sodium in anhydrous ether, followed by carbonation. Mayer, *et al.* (3), described the synthesis of thianaphthene-2-carboxylic acid from *o*-mercaptobenzaldehyde and chloroacetic acid. In 1950 Shirley and Cameron (4) described the synthesis of the 2-carboxylic acid by metalation of thianaphthene with *n*-butyllithium to form 2-thianaphthenyllithium which was then converted into the desired acid by carbonation and acidification.

The thianaphthene-2-carboxylic acid used in this investigation was prepared by a modification of the method of Shirley and Cameron (4). These modifications included conducting the reaction under an atmosphere of nitrogen, and controlling the temperature from −20° to 0° for the formation of *n*-butyllithium and from 0° to 20° for the reaction of *n*-butyllithium with thianaphthene.

EXPERIMENTAL PART I

Thianaphthene-2-carboxylic acid.—A chilled, ethereal solution of 48.04 Gm. (0.75 mole) of *n*-butyllithium, prepared according to the procedure of Gilman, *et al.* (5), was placed in a three-necked flask fitted with a mercury stirrer, dropping funnel, nitrogen inlet, and a low temperature thermometer. Sixty-seven Gm. (0.5 mole) of thianaphthene[1] was dissolved in 100 ml. of anhydrous ether and added dropwise from the dropping funnel to the chilled *n*-butyllithium solution After addition of the thianaphthene solution, the mixture was stirred for two more hours. The internal temperature of the reaction mixture was maintained at 0° to 20° with a freezing mixture of solid carbon dioxide and acetone. The ethereal solution of 2-thianaphthenyllithium was then poured over a slurry of solid carbon dioxide and anhydrous ether. After the carbon dioxide had evaporated, the mixture was diluted with 1,000 ml. of water. The aqueous layer was separated and made acid to Congo Red with concentrated hydrochloric acid. The mixture was allowed to stand overnight at room temperature to insure sufficient coagulation of the acid to permit filtration. The precipitated acid was then separated by filtration, dried and recrystallized from dilute methanol. The air-dried acid yielded 55 Gm. or 62% of the theoretical. M. p. 236°.

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[1] The thianaphthene used in this investigation was supplied through the courtesy of the Texas Company, Beacon, N. Y.

Substituted thianaphthene - 2 - carboxamides.—The general reaction procedure can be illustrated by the synthesis of N-benzylthianaphthene-2-carboxamide. In a 500-ml. three-necked flask, fitted with a mechanical stirrer, dropping funnel, and reflux condenser, was placed 8.9 Gm. (0.05 mole) of thianaphthene-2-carboxylic acid. Forty-one Gm. (0.34 mole) of thionyl chloride was added dropwise and the mixture refluxed at 80° for one hour. The excess thionyl chloride was removed by distillation under reduced pressure. The resultant acid chloride was dissolved in 15 ml. of anhydrous pyridine; then 10.7 Gm. (0.1 mole) of benzylamine, dissolved in 15 ml. of anhydrous pyridine, was added dropwise at room temperature. The reaction mixture was refluxed for three hours and then poured into 300 ml. of water. The precipitated amide was separated by filtration, dried and recrystallized from a mixture of equal parts of acetone and ethyl alcohol. The purified amide was then dried in vacuum at 70°. The yield was 11.4 Gm. or 85% of the theoretical. M. p. 142–143°. *Anal.* Calcd.: N, 5.26%; Found: N, 5.15%.

The conditions of the reactions for the preparation of the compounds in this investigation are given in Table I. Method A involved the addition of the amine at room temperature while in method B the reaction vessel was cooled by an ice-salt freezing mixture during the addition of the amine.

EXPERIMENTAL PART II

The compounds prepared in this investigation were screened for their antifungal activity by a modification of the paper disk technique similar to that reported by Collins and Wiese (6). The fungi used were: *Microsporum gypseum*, *Microsporum canis*, *Trichophyton rubrum*, and *Trichophyton mentagrophytes*. Sabouraud's dextrose agar, pH 5.6, was used as the culture medium. Aqueous suspensions of spores of the test organisms were prepared from fourteen day agar slant cultures which had been incubated at room temperature. The suspensions were prepared by adding 20 ml. of sterile water for injection to the agar slant, grinding the colony growth with the aid of a sterile glass stirring rod, and transferring the suspension to a wide mouth glass bottle containing small glass beads. The colony growth was pulverized into a homogeneous suspension by agitation of the glass container. The culture plates were prepared by pouring 20 ml. of the sterile culture medium into culture plates which were previously sterilized by heating in an oven at 140° for four hours. After solidification of the agar, the plates were inoculated with 1 ml. of the aqueous spore suspension.

The testing procedure consisted of immersing the paper disks into an acetone solution of the compound for thirty seconds and then draining off the excess liquid by touching the disk to the walls of the container. The disks were placed on each culture plate and duplicate plates were run for each compound. The plates were then incubated at room temperature for fourteen days and the zones of inhibition recorded. The zones of inhibition were measured as the minimum distance between the periphery of the paper disk and the colony growth. Control plates containing the medium and acetone only were included in each of the tests. The *in vitro* activity of the compounds was compared to the activity of three known antifungal agents: undecylenic acid, salicylanilide, and 5-chloro-8-hydroxyquinoline.

A preliminary screening of all of the compounds was conducted against *Trichophyton rubrum* and the results of this initial testing are recorded in Table II. Only those compounds which appeared to be equal to or more active than undecylenic acid were tested against the other fungi. The results of these experiments are given in Table III.

TABLE I.—SUBSTITUTED THIANAPHTHENE-2-CARBOXAMIDES

No.	Amine Component	Method	Period of Refluxing, Hr.	Recrystallization Solvent	Yield, %	M. p.	N, Calcd.	N, Found
1.	*p*-Chloroaniline	A	3	Acetone-ethanol	89	208–209	4.87	4.88
2.	*m*-Chloroaniline	A	3	Ethanol	54	165–166	4.87	4.83
3.	*o*-Chloroaniline	A	3	Ethanol	62	131	4.87	4.81
4.	2,4-Dichloroaniline	A	3	Acetone-ethanol	86	145	4.35	4.38
5.	2,5-Dichloroaniline	A	3	Acetone-ethanol	90	135–136	4.35	4.29
6.	Morpholine	B	3	Ethanol	47	99–99.5	5.66	5.53
7.	Benzylamine	A	3	Acetone-ethanol	85	142–143	5.26	5.15
8.	Cyclohexylamine	B	3	Acetone-ethanol	79	161–162	5.40	5.38
9.	Allyl amine	B	3	Ethanol	82	118	6.44	6.37
10.	*p*-Hydroxyaniline	A	6	Acetone-ethanol	66	229–230	5.20	5.13
11.	2-Methylpiperidine	B	3	Ethanol	70	83	5.40	5.32
12.	Phenylethylamine	A	3	Ethanol	76	123	4.97	4.94
13.	*n*-Hexylamine	B	3	Ethanol	86	58–59	5.35	5.26
14.	*m*-Toluidine	A	4	Acetone-ethanol	78	156	5.24	5.10
15.	*o*-Anisidine	A	4	Ethanol	78	121.5–122	4.94	4.87
16.	*p*-Anisidine	A	4	Ethanol	77	186	4.94	4.88
17.	*p*-Toluidine	A	4	Ethanol	74	165–166	5.24	5.04
18.	Piperidine	B	3	Ethanol	85	125	5.70	5.53
19.	2-Aminopyridine	A	3	Ethanol	78	133–133.5	11.02	11.15
20.	Ammonia	B	1	Ethanol	86	176	7.90	7.86
21.	Aniline	A	2	Ethanol	62	188–189	5.53	5.49

TABLE II.—ZONES OF INHIBITION IN MILLIMETERS OF THE RADII SEGMENTS AGAINST T. RUBRUM

Amine Component	0.25%	0.5%	1.0%	2.0%	4.0%
p-Chloroaniline	3	3	3.5	4	5
m-Chloroaniline	1	1	1	1	1
o-Chloroaniline	2	2	2	2	2
2,4-Dichloroaniline	0	0	0	0	0
2,5-Dichloroaniline	2	2	2	2	2
Morpholine	2	3	5	7	9
Benzylamine	2	2	2	2	2
Cyclohexylamine	12	12	12	12.5	15
Allyl amine	4	4.5	6	6	9
p-Hydroxyaniline	0	0	0	0	0
2-Methylpiperidine	4	7	7	7	7
Phenylethylamine	7	7.5	7.5	9.5	10
n-Hexylamine	5	6	7.5	8	9
m-Toluidine	5	5	6	6	7
o-Anisidine	0	0	0	0	0
p-Anisidine	1.5	2	3	5	6
p-Toluidine	5	5	6	6	6
Piperidine	0	0	0	0	0
2-Aminopyridine	3	3.5	4	4	4
Ammonia	1.5	2.5	3	6	6
Aniline	0	0	0	0	0
Thianaphthene-2-carboxylic acid	0	0	0	1	1
Undecylenic acid	0	0	2.5	4.5	4.5
5-Chloro-8-hydroxyquinoline	18	20	31	31	34
Salicylanilide	2.5	3.5	4	4	4
Acetone	0	0	0	0	0

TABLE III.—ZONES OF INHIBITION IN MILLIMETERS OF THE RADII SEGMENTS

Amine Component	%, w/v	*M. gypseum*	*M. canis*	*T. rubrum*	*T. mentagrophytes*
p-Chloroaniline	0.25	0	0	3	0
	0.5	0	0	3	0
	1.0	0	0	3.5	0
	2.0	0	0	4	0
	4.0	0	0	5	0
Morpholine	0.25	0	2.5	2	0
	0.5	0	3	3	0
	1.0	0	3	5	2
	2.0	2	4	7	5
	4.0	6	5	9	7
Cyclohexylamine	0.25	1	3	12	1
	0.5	1	5	12	2
	1.0	1	6	12	3
	2.0	1	6	12.5	3
	4.0	2	7	15	4
Allyl amine	0.25	0	2	4	0
	0.5	0	3	4.5	0
	1.0	0	3.5	6	0
	2.0	0	3.5	6	0
	4.0	1	4	9	0
2-Methylpiperidine	0.25	4	6	4	4.5
	0.5	4	7	7	7
	1.0	4	7	7	7
	2.0	4	8	7	7.5
	4.0	5	8	7	8
Phenylethylamine	0.25	1	2.5	7	2.5
	0.5	1	3.5	7.5	2.5
	1.0	1	6	7.5	2.5
	2.0	1	6	9.5	2.5
	4.0	1	7	10	3
n-Hexylamine	0.25	1	2	5	1
	0.5	1	2	6	2
	1.0	1	2.5	7.5	2
	2.0	1	2.5	8	3.5
	4.0	2	3	9	4
m-Toluidine	0.25	0	1	5	0
	0.5	0	1	5	0
	1.0	0	1	6	0
	2.0	0	1	6	0
	4.0	0	2	7	0

Amine Component	%, w/v	*M. gypseum*	*M. canis*	*T. rubrum*	*T. mentagrophytes*
p-Anisidine	0.25	0	0	1.5	0
	0.5	0	0	2	0
	1.0	0	0	3	0
	2.0	0	1	5	0
	4.0	0	1	6	0
p-Toluidine	0.25	0	1	5	0
	0.5	0	1	5	0
	1.0	0	1	6	0
	2.0	0	1	6	0
	4.0	0	2	6	0
2-Aminopyridine	0.25	0	0.5	3	0
	0.5	0	1	3.5	0
	1.0	0	1	4	0
	2.0	0	2	4	0
	4.0	0	2	6	0
Ammonia	0.25	0	0	1.5	0
	0.5	0	0	2.5	0.5
	1.0	0	0	3	1
	2.0	0	1	6	3
	4.0	2	2	6	4.5
Undecylenic acid	0.25	0	0	0	0
	0.25	0	1	0	0
	1.0	0	1	2.5	0
	2.0	0	4	4.5	0
	4.0	1	4	4.5	1
5-Chloro-8-hydroxyquinoline	0.25	3	9	18	6
	0.5	9	16	20	12
	1.0	12	22	31	18
	2.0	20	23	31	22
	4.0	24	24	34	24
Salicylanilide	0.25	0	4	2.5	2
	0.5	3.5	6	3.5	4
	1.0	5.5	7	4	4
	2.0	6	8	4	6
	4.0	7	10	4	7
Acetone	100.0	0	0	0	0

DISCUSSION

The major impediment in the synthesis of the compounds occurred in the preparation of thianaphthene-2-carboxylic acid. The critical step in the synthesis proved to be the temperature of the reaction between *n*-butyllithium and thianaphthene. It was found that maintenance of an internal temperature of 0° to 20° was essential in order to form 2-thianaphthenyllithium. If the temperature was allowed to rise to the boiling point of the ethereal solvent, an intermediate was formed which when carbonated did not yield the desired acid. The true chemical identity of the compound has not been established but on the basis of its analysis, the empirical formula is $C_{14}H_{10}OS_2$.

The preparation of the individual substituted amides involved determining the optimum conditions for each synthesis and included: 1. variation of the temperature during the addition of the amine, 2. determination of the period of refluxing required, 3. selecting the appropriate solvent for purification.

Thianaphthene-2-carboxamide, N-phenylthianaphthene-2-carboxamide, and N-(2-pyridyl)-thianaphthene-2-carboxamide have been reported in the literature (4) but were included in this investigation in order to relate their antifungal properties with those of the other compounds in this study.

The results of the antifungal studies, as shown in Tables II and III, indicate that none of the compounds were very active as compared to 5-chloro-8 hydroxyquinoline. However, twelve of the compounds tested were equal to or more active than either undecylenic acid or salicylanilide against *Trichophyton rubrum*.

Only those compounds which were equal to or more active than undecylenic acid against *Trichophyton rubrum* were screened against the other organisms. Seven of the compounds appeared to be equal to or more active than undecylenic acid against *Microsporum gypseum;* five of the compounds were equal to or more active than undecylenic acid against *Microsporum canis* and six of the compounds were equal to or more active than undecylenic acid against *Trichophyton mentagrophytes*. N-(2-Methylpiperidyl)-thianaphthene-2-carboxamide, N-cyclohexylthianaphthene-2-carboxamide, and N-morpholinylthianaphthene-2-carboxamide were the most active compounds against all four of the fungi tested.

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A Study of the Selective Antagonism by N-Allylnormorphine Against Meperidine, Methadone, and Morphine*

By THOMAS A. LYNCH and DONALD B. MEYERS

A study has been made of the selective antagonism by N-allylnormorphine against meperidine, methadone, and morphine. The degree of protection offered by 10 mg./Kg. of N-allylnormorphine against the acute toxicity of the narcotics in mice and rats has been determined. The extent of antagonism of the narcotic-induced depression of rabbit minute respiratory volume has been determined by repeated fixed doses of N-allylnormorphine. Further work on rabbit respiration has been carried out by administration of different mixed doses of narcotic and antagonist. Tests of selective antagonism against convulsive and acute toxic effects of the narcotics in frogs have been made.

THERE IS AMPLE EVIDENCE of the specific antagonistic effect of N-allylnormorphine against the pharmacological actions of morphine and the other chemically related compounds (1–7). However, selectivity of this antagonism toward the various narcotics has not been quantitatively studied. Such a consideration may be important in determining the relative effectiveness of N-allylnormorphine in conjunction with clinically used narcotics, in elucidating the mechanism of action of the narcotics, and in establishing a suitable procedure for evaluating other N-allyl derivatives. This investigation was undertaken to determine if a selectivity in antagonism exists.

EXPERIMENTAL

Protection Against Acute Toxicity of Narcotics in Mice.—In this study 500 albino mice of mixed sex weighing 15 to 25 Gm. were used. The acute toxicity of meperidine, methadone, and morphine was determined in untreated mice, and in mice pretreated with 10 mg./Kg. of N-allylnormorphine fifteen minutes prior to the injection of the narcotic. All injections were made by the subcutaneous route. The LD_{50}'s and per cent limits of error were determined according to the method of deBeer (8).

Protection Against Acute Toxicity of Narcotics in Rats.—One hundred seventy-six albino rats of mixed sex weighing 250 to 450 Gm. were used. The acute toxicity of meperidine, methadone, and morphine was determined in untreated rats, and in rats pretreated with 10 mg./Kg. of N-allylnormorphine fifteen minutes prior to the injection of the narcotic. All injections were made by the intraperitoneal route. The LD_{50}'s and per cent limits of error were determined as with the mice.

Antagonism of Respiratory Depression by Narcotics in Rabbits.—The minute respiratory volume of the unanesthetized rabbit was measured by use of a spirometer. The displacement of the movable cylinder was recorded on a smoked kymograph by a stylus attached to the cylinder. Comparison of minute respiratory volumes was based on the average normal which was expressed as 100%. Injections were made into the marginal ear vein.

Method I.—Antagonism of narcotic-induced respiratory depression was determined by repeated fixed doses of N-allylnormorphine. After 3 to 5 normal consistent minute respiratory volumes were recorded at two minute intervals, a dose of the narcotic was given which depressed the respiration. Then single doses of N-allylnormorphine were administered at three minute intervals until the respiration returned to normal. The total dosage of antagonist required to restore the respiration to normal was expressed in a ratio relationship to the narcotic.

Method II.—The antagonism by N-allylnormorphine was shown by the administration of mixtures of the antagonist and narcotic. After the normal respiration was recorded, various mixture ratios were administered and the minute respiratory volume recorded 3 times at five minute intervals. Then double the previous dose of the same ratio was given, and the respiration recorded 3 times at five minute intervals. The averages of each of the 3 recordings were expressed in a percentage relationship to the average normal.

Effect of N-Allylmormorphine Upon Convulsions and Acute Toxicity by Narcotics in Frogs.—Frogs of the species, *Rana pipiens*, weighing 20 to 40 Gm. were administered the drugs by way of the ventral lymph sac. After induction of convulsions in frogs by a minimal dose of the narcotic, N-allylnormorphine (10 mg./Kg.) was administered.

The acute toxicity of meperidine, methadone, and morphine was determined in 120 frogs. The LD_{50}'s and per cent limits of error were determined as previously, after which, 1 LD_{50} of each narcotic was administered to groups of 24 frogs pretreated with 10 mg./Kg. of N-allylnormorphine fifteen minutes previously. The per cent mortality was noted.

RESULTS AND DISCUSSION

Antagonism of Acute Toxicity in Mice and Rats.—The acute subcutaneous toxicities of meperidine, methadone, and morphine in untreated mice and mice pretreated with N-allylnormorphine are given in Table I; similarly, the acute intraperitoneal toxicities in rats are given in Table II.

* Received April 16, 1956, from the College of Pharmacy, Butler University, Indianapolis, Ind.

TABLE I.—ACUTE SUBCUTANEOUS TOXICITIES OF NARCOTICS IN MICE UNTREATED AND PRETREATED WITH N-ALLYLNORMORPHINE (10 MG./KG.)[a]

Compound	LD_{50} (Per cent Limits of Error) Untreated mg./Kg.	Pretreated mg./Kg.
Meperidine	112 (95.5–105.5)	213 (91–110)
Methadone	38 (73–137)	94 (98–102)
Morphine	390 (87–115)	470 (94–107)

[a] N-Allylnormorphine was given subcutaneously.

TABLE II.—ACUTE INTRAPERITONEAL TOXICITIES OF NARCOTICS IN RATS UNTREATED AND PRETREATED WITH N-ALLYLNORMORPHINE (10 MG./KG.)[a]

Compound	LD_{50} (Per cent Limits of Error) Untreated mg./Kg.	Pretreated mg./Kg.
Meperidine	87 (95–106)	104 (85.5–117)
Methadone	18 (79–127)	40 (90–110)
Morphine	201 (92–109)	...[b]

[a] N-Allylnormorphine was given intraperitoneally.
[b] LD_{50} could not be determined due to erratic response.

The extent of selective antagonism by N-allylnormorphine was evaluated by comparing the number of micromoles of narcotic protected by 1 micromole of N-allylnormorphine as shown in Table III.

TABLE III.—PROTECTION BY N-ALLYLNORMORPHINE AGAINST THE LD_{50} OF NARCOTICS AS EXPRESSED BY THE MOLAR RELATIONSHIP

Compound	Limits of Protection,[a] micromoles/Kg.	Mean Protected Dose,[b] micromoles/Kg.
	Mice	
Meperidine	259–365	10.8
Methadone	129–204	5.8
Morphine	0–441	7.7
	Rats	
Meperidine	0–139	2.4
Methadone	38– 87	2.2

[a] Protection by 28.8 micromoles/Kg. of N-allylnormorphine, the difference of LD_{50}'s of pretreated and untreated animals.
[b] Protection by 1 micromole/Kg. of N-allylnormorphine.

The limits of protection were derived from the per cent limits of error of the LD_{50}'s. The lower and upper limit of each LD_{50} was calculated by multiplying the LD_{50} by its lower and upper per cent limits of error. The lower limit of protection was determined by subtracting the upper limit of the LD_{50} of the untreated animals from the lower limit of the LD_{50} of the pretreated animals. The upper limit of protection was found by subtracting the lower limit of the LD_{50} of the untreated animals from the upper limit of the LD_{50} of the pretreated animals. All doses were expressed as the molar equivalents. The mean protected dose by 1 micromole/Kg. of N-allylnormorphine shows that protection is greatest for meperidine and the least for methadone in mice, while protection was only slightly greater for meperidine than methadone in rats. This relative difference in protection of the two species may be due to the difference in the ratio of the antagonist to the narcotic, since the optimum antagonistic effect may depend on this ratio, as suggested by Hart and McCawley (2) using morphine.

Antagonism of Rabbit Respiratory Depression.—Repeated Fixed Dose Antatonism.—Table IV presents the results of antagonism by repeated fixed doses of N-allylnormorphine against methadone and morphine. The average total dosage of N-allylnormorphine was expressed in a ratio relationship to the respiratory depressant dose of the narcotic. Quantitative antagonism by meperidine could not be determined by this method, since the respiratory depression produced was comparatively less pronounced and more transient than by either methadone or morphine. Morphine at a dose of 4 mg./Kg. produced an average of 52.6% depression of the normal respiration, which is approximately the same as that produced by 0.75 mg./Kg. of methadone.

TABLE IV.—REPEATED FIXED DOSE ANTAGONISM OF METHADONE AND MORPHINE RESPIRATORY DEPRESSION IN RABBITS BY N-ALLYLNORMORPHINE

Compound	No. of Rabbits	Dose, mg./Kg.	Av. % Depression	Dose of[a] NAM, µg./Kg.	Ratio[b]
Methadone	2	1.5	73.8	19	1:81
	15	1.0	66.0	16	1:74
	5	0.75	54.9	19	1:41
	2	0.50	49.5	29	1:19
Morphine	13	4.0	52.6	54	1:48
	3	3.0	53.2	91	1:33

[a] Average total dosage of N-allylnormorphine (NAM) necessary to restore normal minute volume.
[b] Ratio of total dosage of N-allylnormorphine to narcotic.

The average ratios of N-allylnormorphine necessary to restore the respiration to normal at the respective depressant doses of narcotics are as follows: morphine, 1:48 and methadone, 1:41. Upon conversion of these ratios to their molar equivalents, they compare as follows: morphine, 1:43.9 and methadone, 1:41.3. These results indicate very slight selective antagonism by N-allylnormorphine for morphine.

Antagonism by Mixtures of Narcotic and N-Allylnormorphine.—Comparison of the antagonism by simultaneous administration of narcotic and N-allylnormorphine is shown in Table V. The dose of the narcotic in each mixture was kept constant as follows: methadone (0.75 mg./Kg.); morphine (4.0 mg./Kg.); and meperidine (5.0 mg./Kg). The subsequent second dose contained twice the amount of narcotic but was the same ratio as the first dose. The mixture ratios of antagonist to narcotic which approximated an equivalent response to near normal respiration are as follows: meperidine, 1:40; morphine, 1:10; and methadone, 1:5. Therefore, it seems that respiratory depression by meperidine is antagonized most effectively and methadone the least effectively.

The validity of comparing the meperidine response with that of methadone and morphine may seem questionable, since meperidine did not produce the equivalent respiratory depression. It was found that the mixture of the ratio of antagonist and narcotic was more important than the quantity of mixture administered, for the dose of the mixture in each case could be doubled without appreciable de-

TABLE V.—EFFECTS OF MIXTURE OF NARCOTICS AND N-ALLYLNORMORPHINE ON MINUTE RESPIRATORY VOLUME OF RABBITS

Compound	No. of Recordings	Ratio[a]	Av. Respiratory Depression, %—1st dose	Av. Respiratory Depression, %—2nd dose[b]
Methadone	4	1:60	58.7	..
	2	1:20	38.4	64.7
	9	1:15	29.7	36.2
	7	1:10	13.1	16.8
	6	1:5	12.5	11.9
Morphine	2	1:80	62.5	63.7
	6	1:30	32.5	33.9
	6	1:20	17.9	19.4
	6	1:10	6.8	8.7
Meperidine	6	[c]	14.0	26.5
	4	1:40	6.0	9.0
	6	1:20	0.4	13.0[d]
	1	1:15	9.0[d]	29.0[d]
	1	1:5	7.7	33.0[d]

[a] Ratio of N-allylnormorphine to narcotic.
[b] Two times the amount of narcotic as the first dose.
[c] Meperidine was administered alone.
[d] Increase per cent of normal respiration.

viation from the response of the first dose as suggested by the data in Fig. 1. The lack of conformity of the meperidine curves does not contradict the above statement, since the curves above the 100% of normal respiration are due possibly to synergism of convulsiveness rather than antagonism. The results show the same qualitative relationship of selectivity as obtained with acute toxicity in mice. These results are probably more representative of the true antagonistic effect than those obtained by the repeated dose method, for the narcotic and antagonist in this case were allowed to act simultaneously. Also, the variability of the repeated dose method was eliminated.

Relationship of N-Allylnormorphine to Narcotics in Frogs.—Convulsions.—In an effort to determine narcotic antagonism in frogs, it was found that N-allylnormorphine (10 mg./Kg.) failed to prevent convulsions induced by meperidine (250 mg./Kg.) and morphine (500 mg./Kg.), while convulsions could not be produced by methadone.

Acute Toxicity.—The acute intralymphatic toxicity of meperidine, methadone, and morphine in frogs was determined, after which, 1 LD_{50} of these compounds were administered to frogs pretreated fifteen minutes before with N-allylnormorphine (10 mg./Kg.). As shown in Table VI, N-allylnormorphine increased the acute toxicity of methadone and meperidine in reverse order of the size of the LD_{50}. The effect upon toxicity may be attributed to synergism of N-allylnormorphine and narcotic on the spinal reflexes of the frog, in light of the fact that N-allylnormorphine is known to produce hyperactivity of the hindlimb reflexes of the chronic spinal dog, as reported by Wikler and Carter (9).

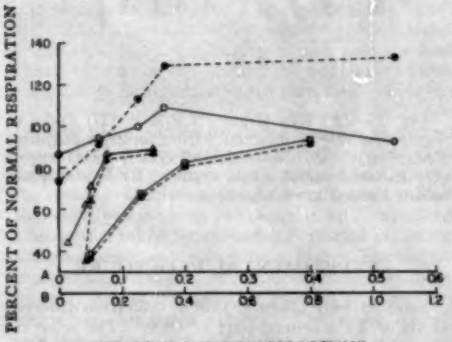

Fig. 1—Effect of different mixtures of narcotic and N-allylnormorphine upon rabbit respiratory volume.

○ Meperidine (5 mg./Kg.); △ Methadone (0.75 mg./Kg.); □ Morphine (4 mg./Kg.); ● Meperidine (10 mg./Kg.); ▲ Methadone (1.5 mg./Kg.); ■ Morphine (8 mg./Kg.); ——A, - - - -B.

TABLE VI.—EFFECT OF N-ALLYNORMORPHINE (10 MG./KG.)[a] PRETREATMENT ON THE ACUTE INTRALYMPHATIC TOXICITY OF NARCOTICS IN FROGS

Compound	LD_{50}[b] (% Limits of Error), mg./Kg.	No. of Frogs	% Mortality
Meperidine	191(97–104)	24	66.7
Methadone	67(97–103)	24	100.0
Morphine	590(64–150)	24	54.1

[a] N-Allylnormorphine was given intralymphatically fifteen minutes prior to the narcotic.
[b] LD_{50} of untreated frogs and the dose given intralymphatically to pretreated frogs.

SUMMARY

1. The selective antagonism of meperidine, methadone, and morphine by N-allylnormorphine has been determined.

2. N-Allylnormorphine exhibited greatest selectivity of antagonism for meperidine and the least for methadone in protecting against acute toxicity in mice. Selective antagonism of meperidine was not significantly greater than methadone in rats.

3. Antagonism was most selective for meperidine and the least selective for methadone on rabbit respiration by the mixture method, while little selectivity was shown by the repeated dose method.

4. Antagonism by N-allylnormorphine could not be shown against the convulsions or acute toxicity in frogs; on the contrary, acute toxicity of methadone and meperidine was augmented.

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A Study on Couch Grass Ergot*

By LEE C. SCHRAMM† and JACK L. BEAL

Ergot found growing on couch grass in Delaware and Franklin Counties in the central part of Ohio was found to contain above N. F. X limits in ergotoxine content, and below N. F. X limits in ergonovine content. It was also noted that part of the sample was infested with a small beetle-like insect. The infested portions assayed lower in alkaloid and fat content. Evidence was presented by paper chromatography to show the presence of a number of ergot alkaloids and lysergic acid.

Claviceps purpurea (Fries) Tulasne is a parasitic fungus commonly occurring on rye. Ergot of commerce is defined as the sclerotium, or resting stage, of *Claviceps purpurea*—a hard, purplish, somewhat curved body, the size of which is usually determined by the size of the grain it parasitizes. It has been noted that *Claviceps purpurea* infects other members of the *Gramineae* (grass family) (1), particularly the cereal grasses.

In the summer of 1956, it was noticed that there was a local infection of *Agropyron repens* (Linne) Beauvois (couch grass, quack grass, triticum) with *Claviceps purpurea*. Couch grass was noted to grow along roads and highways throughout the central part of Ohio, usually along the fence bordering a wheat field. It was also noted that the wheat adjacent to the infected couch grass was infected with ergot. Couch grass, a member of the cereal grasses, is a perennial weed, the aerial portion consisting of a leafy simple culm attaining the height of 1 meter, and bearing a terminal spike of many spikelets (2). The rhizome of this grass, known as Triticum, has been used as a demulcent diuretic, and was official in the U. S. P. from 1885 to 1926, and the N. F. from 1926 to 1950.

Since a survey of the literature failed to disclose any pharmacognostical studies of *Claviceps purpurea* found growing on couch grass, it was decided that an investigation should be made. This investigation includes the determination of the fat and moisture content, the determination of total alkaloid content, and a study of the individual alkaloids contained therein.

On further study of the individual sclerotia, it was found that about one-half of the sample was infested by a small beetle; thus, another division of the investigation was made, that is, to determine what effect, if any, this infestation had on the fat, moisture, and alkaloid content.

The identity of the fungus, as *Claviceps purpurea* and the grass as *Agropyron repens* was verified by the botany department of The Ohio State University.[1]

The average size of the sclerotia was found to be 10–12 mm. in length (ranging from 6 to 18 mm.) and 1–2 mm. in width. The size relative to rye ergot may be observed in Fig. 1.

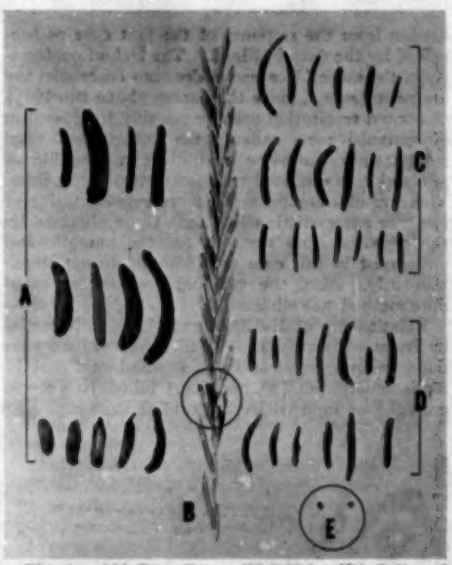

Fig. 1.—(A) Rye Ergot (N.F.X.); (B) Spike of *Agropyron repens* infected with *Claviceps purpurea* (encircled); (C) Non-infested couch grass ergot; (D) Infested couch grass ergot; (E) Beetles from infested couch grass ergot (encircled).

EXPERIMENTAL PROCEDURE

Samples were taken in July of 1956 from different sections of the central part of Ohio. The sclerotia were separated from the grains, and then subdivided into two portions, the noninfested and the infested portions. The two portions were then independently passed through a Wiley Laboratory Mill and ground to a No. 20 powder.

* Received September 23, 1957, from the College of Pharmacy, Ohio State University, Columbus.
† Recipient of the Kilmer Prize, 1957.

[1] The authors express their appreciation to Dr. John A. Schmitt, Department of Botany and Plant Pathology, Ohio State University, for verifying the identity of the fungus.

Moisture Determination.—The moisture determination was carried out according to the N. F. method (3). The results are listed in Table I.

Fat Determination.—Three 500-mg. samples of each portion of the ground drug were extracted by continuous extraction in Soxhlet apparatus with petroleum ether for twenty hours. The extracted matter was then transferred to a tared evaporating dish, the extraction apparatus rinsed with three small portions of the solvent, the rinsings added to the extracted matter, and the solvent evaporated to dryness over low heat. The dish was then dried in an oven at 105° to a constant weight. The amount of residue was then determined and the per cent total fat calculated from the data. The results are listed in Table I.

TABLE I.—RESULTS OF ANALYSIS

	Noninfested	Infested
Moisture,[a] %	9.2	9.2
Fat,[a] %	15.9	13.3
Alkaloids[b]		
Water-insoluble,[c] mg. %	198.05	177.86
Water-soluble,[d] mg. %	3.93	3.19

[a] Average of 3 samples.
[b] Average of 4 samples expressed as moisture and fat free.
[c] Calculated as ergotoxine (3).
[d] Calculated as ergonovine (9).

Alkaloid Determination.—The alkaloid determination was carried out according to the method of Silber and Schulze (4) with modifications. Four 200-mg. samples of both portions of the defatted material were extracted by means of continuous extraction in Soxhlet apparatus with a mixture of 0.1 ml. 10% ammonia solution and 6 ml. ether U. S. P. per 100 mg. of sample. Extraction was continued to exhaustion, which was experimentally shown to be approximately six hours. The percolate was removed, the apparatus washed with three successive 3-ml. portions of ether, placed in a 25-ml. flask, and concentrated to 5 ml. at a temperature not above 40°. This concentrated percolate was then transferred to a 30-ml. separatory funnel, and the flask rinsed with three successive 1-ml. portions of ether, the rinsings being added to the percolate. This was then shaken first with three successive 3-ml. portions of pH 8 phosphate buffer solution to extract the water-soluble alkaloids, and then with three successive 3-ml. portions of 2% tartaric acid solution to remove the water-insoluble alkaloids. Each shake-out was made up to exactly 10 ml., and a 2-ml. aliquot was taken for the assay. Each 2 ml. of unknown sample was mixed in a Fisher Electrophotometer Cell with 4 ml. of *p*-dimethylaminobenzaldehyde T. S. and immediately exposed to a mercury vapor lamp for seven minutes. The optical density was then determined in a Fisher Electrophotometer at 595μ. For each individual determination, a blank was prepared using 2 ml. of 2% tartaric acid solution and 4 ml. of *p*-dimethylaminobenzaldehyde T. S., illuminated for the same length of time, and the meter adjusted to zero optical density.

A reference graph was prepared using U. S. P. reference standard ergonovine maleate in concentrations ranging from 2 to 100 μg. per ml. in 1:1,000 tartaric acid solution. The same procedure as was used in the assay was followed in determining the values for the reference graph. The results of the assay are listed in Table I. The illuminating lamp was of the mercury-vapor type as is supplied with the Lumetron colorimeter.

The *p*-dimethylaminobenzaldehyde reagent was prepared after the U. S. P. XV (5) and the phosphate buffer solution was prepared after Silber and Schulze (4).

Identification.—Identification of the water-insoluble fraction of the alkaloids was carried out by means of descending paper partition chromatography, using the tartaric acid solutions obtained in the assay. The solutions were made alkaline with ammonia T. S. and extracted with ether. Wide sheets of Whatman No. 1 paper (7½ inch x 18 inch) were used for this experiment, since multiple separations could be run concurrently on the same paper, saving time, and eliminating variations in degree of saturation of the chamber. The papers were saturated with formamide, accomplished by passing them through a solution of acetone-formamide 3:1 and allowing the excess solvent to evaporate. The solutions of the alkaloids were applied to the starting line with a micro-pipet in 20 μl. amounts. The starting line was about 4 inches from the end of the paper to allow the solvent front to become equable as it crossed the line (6). The mobile phase consisted of benzene saturated with formamide, and the paper was developed in a previously saturated chamber (7). Development required approximately three hours; allowing the solvent front to run ¾ to ⅞ the length of the paper. The results are listed in Table II.

TABLE II.—R_f TABLE USING THE BENZENE-FORMAMIDE PROCEDURE FOR WATER-INSOLUBLE ALKALOIDS

Alkaloids	R_f Values by Kolsek (7)	Range of R_f Values of N. F. Ergot[a]	Range of R_f Values of Couch Grass Ergot[a]
Ergotamine	0.11	0.08–0.11	0.08–0.09
Ergosine	0.13	0.12–0.16	0.14–0.16
Ergotaminine	0.40	0.40–0.45	0.39–0.41
Ergosinine	0.48	0.48–0.55	0.50–0.53
Ergocornine	0.52	0.54–0.63	0.58–0.62
Ergocristine			
Ergocryptine	0.62	0.65–0.76	0.69–0.72
Ergocorninine			
Ergocristinine	0.86	0.81–0.90	0.79–0.85
Ergocryptinine			
Lysergic Acid	0.00	0.00	0.00

[a] Represents 12 determinations.

Identification of the water-soluble fraction of the alkaloids was carried out by the method of Tyler and Schwarting (8) using the buffer solutions obtained in the assay. The mobile phase consisted of the upper layer of a normal butanol-glacial acetic acid-water mixture 4:1:5. The aqueous alkaloid solutions were applied to the starting line in 40 μl. amounts to insure concentrations of the alkaloids adequate for detection. Control spots were used along with the unknown spots, consisting of reference standard ergonovine maleate in 2% tartaric

acid solution in a concentration of 200 μg. per ml., applied in 20-μl. amounts. Development time was considerably longer, requiring approximately six to twelve hours, the longer time being more desirable. The results are listed in Table III.

TABLE III.—R_f TABLE USING THE *n*-BUTANOL-ACETIC ACID-WATER PROCEDURE FOR WATER SOLUBLE ALKALOIDS

	Range of R_f Values[a]	Average
Ergonovine reference standard	0.60–0.66	0.63
Ergonovine (Couch grass)	0.64–0.66	0.64

[a] Represents 6 determinations.

Since the data in Table II indicate the possible presence of lysergic acid a further check for the presence of lysergic acid was carried out by chromatographing fresh samples of both the tartaric acid fraction and the buffer solution fraction. The procedure was the same as that used for the identification of the water-soluble fraction of the alkaloids. A reference sample of lysergic acid[1] was chromatographed concurrently in the same chamber as that used for the unknown solutions. The results are listed in Table IV.

TABLE IV.—R_f TABLE USING THE *n*-BUTANOL-ACETIC ACID-WATER PROCEDURE FOR CHECKING THE PRESENCE OF LYSERGIC ACID

Sample	Range of R_f Values	Average R_f[a]
Lysergic acid in tartaric acid extract	0.24–0.26	0.24
Lysergic acid reference	0.23–0.27	0.25
Lysergic acid in buffer extract	0.27–0.30	0.29
Lysergic acid reference	0.30–0.33	0.32

[a] Average of 6 determinations.

DISCUSSION

While the moisture content was the same for the infested and noninfested portions, there was a difference in the fat content. The noninfested portion contained approximately 2.5% more fat than the infested. This could be explained on the basis of a liking by the beetle for the lipid portion of the sclerotium, or on the basis of the weight added by the contamination of the sample by the beetle.

Silber and Schulze (4) claimed that a complete extraction of alkaloids could be obtained merely by shaking the ground drug with solvent for two hours, but it was felt that Soxhlet extraction to exhaustion was certain of being complete.

The noninfested ergot showed a larger amount of water-soluble and water-insoluble alkaloids than the infested ergot; the difference in each case followed approximately the same ratio. In both cases the water-insoluble fraction was above the N. F. limit (10) while the water-soluble fraction was below the limit.

[1] The authors thank Eli Lilly and Co. for providing the sample of lysergic acid used in this experiment.

In the paper chromatography experiments for the identification of the individual alkaloids only the noninfested ergot was used.

It is well known that R_f values are modified by many factors, for example, temperature, concentration, and ratio of concentrations. Positive identification of alkaloids is impossible unless the above factors remain constant. It is often desirable to have pure samples of each alkaloid in question to use as R_f reference standards. However, in this case, this would be of little value because the exact ratio of concentrations of the alkaloids of the unknown sample cannot be ascertained. Bearing the above statements in mind, it was believed that the only possible method of identifying the alkaloids on the chromatogram in this experiment was to follow a procedure which accounted for the alkaloids commonly occurring in ergot. Such a procedure was that of J. Kolsek (7) whereby he reported R_f values of the major ergot alkaloids singly and in mixtures.

In one particular experiment, using a known mixture, he reported 7 R_f values which accounted for 10 water-insoluble alkaloids of ergot. In this case, ergocornine and ergocristine were not separated, representing one spot, and ergocorninine, ergocristinine, and ergocryptinine were also not separated, representing one spot. As can be observed in Table II, the R_f values obtained in this experiment were similar but not identical to those of Kolsek. In addition, there was no overlapping in the ranges of R_f values. This similarity is not positive proof of identification; however, in referring to Table II there is evidence that the sequence of identity of the spots would be the same as Kolsek's. For example, in Table II, if Kolsek's 4th spot, R_f 0.48 was ergosine, the 4th spot of couch grass ergot, R_f 0.50–0.53 is also ergosine.

The identification uses nomenclature of the ergotoxine group of alkaloids by Stoll as reported by Manske and Holmes (11), namely that ergotoxine is not homogenous, but a complex mixture of ergocristine, ergocryptine, and ergocornine and may contain their isomers.

The alkaloid of the water-soluble fraction was identified as ergonovine, as is shown in Table III.

The possibility of the presence of lysergic acid is indicated in Table II and Table IV. Kolsek reports (7) that in using the benzene-formamide procedure lysergic acid does not move, thus the R_f is zero. In the water-insoluble alkaloidal fraction of couch grass ergot there was such a spot having a zero R_f as indicated in Table II. Table IV lists the R_f values for the suspected lysergic acid in the water-insoluble alkaloidal fraction as well as in the water-soluble fraction. In each case the R_f for the suspected lysergic acid is very close to that of a known sample of lysergic acid. This similarity of R_f in two different mobile phase systems, the benzene-formamide and the *n*-butanol-glacial acetic acid–water mixture, gives some evidence for the presence of lysergic acid. In each case the spot indicated, by reason of its faintness, only a small amount of the substance to be present. Assuming the substance to be lysergic acid, this study does not determine whether the lysergic acid was originally present in the ergot or whether its origin is due to the extraction procedure.

Since this problem was undertaken, it was brought to our attention that Silber and Bischoff (12) have analyzed ergot of *Agropyrom repens* Pal. Beauvois

and reported total alkaloid content as ergotamine in three different samples. The values were reported as 110, 102, and 375 mg. %. No other determinations were made.

SUMMARY AND CONCLUSIONS

Ergot of couch grass was studied with respect to the fat, moisture, and alkaloid contents. It was found that half of the sample was infested with a beetle, and determinations were extended to find out what effect this infestation had on the contents of the drug. It was found that:

1. The moisture content of both infested and noninfested portions was the same.

2. The fat content of infested ergot was lower than that of the noninfested portion.

3. The alkaloid content of the infested ergot was lower than that of the noninfested portion.

4. Both portions assayed above N. F. limits in ergotoxine content, and below N. F. limits in ergonovine content.

5. Evidence was presented to show that ergotamine, ergotaminine, ergosine, ergosinine, ergocornine, ergocorninine, ergocristine, ergocristinine, ergocryptine, ergocryptinine, and lysergic acid were probably present.

6. Ergonovine was positively shown to be present.

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Decomposition of Aspirin in the Solid State*

By LEWIS J. LEESON† and ALBERT M. MATTOCKS

The decomposition of aspirin in the solid state has been demonstrated to be dependent on vapor pressure and temperature. A possible mechanism of decomposition is offered, which consists of an initial sorption of a water layer by each particle, diffusion of aspirin into solution, and decomposition taking place by acid catalyzed hydrolysis. A series of equations which describe the mechanism have been derived and the experimental data demonstrated to fit them. Using these equations, the various kinetic constants have been determined.

SINCE SOLID DOSAGE FORMS comprise a large segment of the drugs marketed today, there is a great need for methods of predicting their stability under various conditions of storage. In order for such a method to be dependable it must be based upon a knowledge of the mechanisms involved and quantitative studies of the variables affecting the reactions.

Many pharmaceuticals are known to exhibit decomposition in solid form, and perhaps the most widely used one is aspirin. Aspirin is an especially interesting example of this type of reaction since it is known to be affected not only by temperature and humidity but also by numerous chemical agents with which it is often combined.

Decomposition of aspirin in solid form has been noted by Tsakalotos (1), Paolini (2), and Strathopoulos (3). In addition, Ribeiro, *et al.* (4), found that certain lubricants, notably stearates, increased the degree of decomposition of aspirin contained in aspirin-phenacetin-caffeine tablets. Ebert (5), and Yamamoto and Takahashi (6, 7) studied the accelerating effects of certain amines, temperature, humidity, pressure, and grinding upon aspirin loss in tablets. These studies were concerned primarily with effects of various chemical agents and conditions and did not attempt to determine reaction rates or mechanisms.

There have been a number of kinetic studies on aspirin decomposition in solution (8–11), the most complete investigation being reported by Edwards (12). He agreed with other workers that the decomposition is first order with respect to aspirin concentration, but by studying the reaction at various pH values he demonstrated that the apparent first order rate constant is actually a composite of six rate constants. Each

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of the six constants is associated with one of the hydrolytic reactions depending upon hydrogen ion, hydroxyl ion, or water reacting with either molecular or ionic aspirin.

There is no direct method for applying to solid forms information obtained from studies of decomposition of a drug in solution, since it is even questionable whether the same reactions occur. Thus it is important that studies be made of reactions of drugs in solid state, and such a study was undertaken in these laboratories using aspirin as the first example.

EXPERIMENTAL

Aspirin powder of U. S. P. grade (Mallinckrodt) was used for this investigation. It was classified into various sizes by means of U. S. Standard sieves. Extent of aspirin decomposition was determined by measurement of the salicylic acid formed; this was accomplished by a modification of the A.O.A.C. procedure (13). The sample was dissolved in 100 ml. absolute alcohol, a suitable aliquot was transferred to a 100-ml. volumetric flask, and sufficient absolute alcohol was added to make the total alcohol content 50 ml. Two milliliters of ferric ammonium sulfate T. S. was added and the solution was made up to volume with water. The color intensity was measured on a spectrophotometer at 532 mμ.

Since it was not known whether aspirin would decompose if no moisture were present, a preliminary experiment was run in the absence of water vapor. One-half gram samples of aspirin of 100/140 screen size were sealed in ampuls and stored at 35°, 45°, 60°, 80°, 100°, and 110°. Similar samples to which 2.5% calcium stearate had been added were stored under identical conditions. Calcium stearate was added since it has been reported to induce extensive decomposition of aspirin (4). Samples were removed at various times over a period of fifty days and assayed.

Samples of aspirin alone showed little or no decomposition at 80° or below. Those containing calcium stearate decomposed in a short time to the extent of about 1% and then remained constant. In samples stored at 100° and 110° salicylic acid was found to increase rapidly to about 2% and then decrease gradually with time. These samples were observed to melt and change color, and it is not known whether the color interfered with the assay or the decrease in salicylic acid might be due to salicylide formation.

It is believed that the small amount of decomposition detected in these samples could have been caused by traces of moisture which contaminated the dry aspirin during the process of filling the ampuls. The amount of water necessary to account for the decomposition observed is approximately 10^{-4} moles. It was concluded, therefore, that decomposition of aspirin in the absence of moisture is of minor importance. Consequently, attention was directed to the decomposition of aspirin in the presence of water vapor.

A series of samples of aspirin of screen size 100/140, weighing approximately 0.25 Gm., were placed in loosely capped, screw-top vials which were stored at 50°, 60°, and 80°; and at varying humidities obtained by use of saturated inorganic salt solutions (14). At various times samples were removed and analyzed for extent of decomposition. Results are shown in Table I. A graph showing the general shape of the curve relating decomposition to time is shown in Fig. 1.

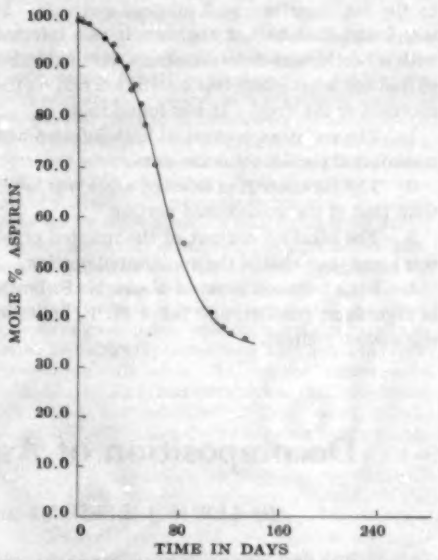

Fig. 1.—Typical decomposition of aspirin in solid state (60°, vapor pressure 120.3 mm.).

In order to be certain that the hydrolysis reaction was the only one of major importance and that no salicylic acid was lost by volatilization, an ultraviolet assay for aspirin and salicylic acid (5) was performed on numerous samples. This method consisted of reading solutions of aspirin and salicylic acid in absolute alcohol at 226 mμ and 235 mμ, which are the absorbance peaks for aspirin and salicylic acid, respectively. The amount of each present was then determined by the usual two-component system technique. In all cases the original amount of aspirin could be accounted for by a summation of the aspirin and salicylic acid found.

TREATMENT OF THE DATA

Examination of the data of Table I by means of typical plots used to determine reaction order made it obvious that the reaction studied did not follow one of the simpler kinetic relationships. The curves obtained, illustrated in Fig. 1, resembled typical autocatalytic plots, but an attempt to establish autocatalysis by graphical means gave negative results. Therefore, a mechanism was postulated based on the known physical and chemical conditions of the reaction.

The proposed mechanism is based on the following tenets: (a) Water is rapidly adsorbed onto the surface of the aspirin, the amount of water being a function of vapor pressure. If multilayer adsorp-

TABLE I.—DECOMPOSITION OF ASPIRIN AT VARIOUS TEMPERATURES AND VAPOR PRESSURES

50°

46.02 mm.		54.40 mm.		68.30 mm.		74.20 mm.	
Time in Days	Mole % Aspirin	Time in Days	Mole % Aspirin	Time in days	Mole % Aspirin	Time in Days	Mole % Aspirin
0.0	99.90	0.0	99.90	0.0	99.90	0.0	99.90
3.0	99.87	3.0	99.86	3.0	99.84	7.0	99.59
24.0	99.83	24.0	99.70	7.0	99.60	16.0	99.32
34.1	99.66	34.0	99.43	21.9	99.22	24.0	99.05
40.9	99.53	40.9	99.27	30.0	99.56	30.0	98.92
47.9	99.29	47.9	98.83	34.1	99.05	34.0	98.76
61.9	99.26	61.7	98.98	40.9	98.97	40.9	98.72
76.8	98.73	76.8	98.26	47.9	98.69	47.9	98.24
103.8	98.51	103.8	97.83	61.9	98.10	61.9	96.72
139.8	97.77	139.8	96.91	76.8	97.40	76.8	96.16
170.8	96.86	170.8	94.69	103.8	95.98	103.8	93.29
...	...	...	...	139.8	93.82	139.8	89.78
...	...	...	...	170.8	92.64	170.8	86.75

60°

68.41 mm.		74.50 mm.		111.9 mm.		120.3 mm.	
Time in Days	Mole % Aspirin	Time in Days	Mole % Aspirin	Time in Days	Mole % Aspirin	Time in Days	Mole % Aspirin
0.0	99.90	0.0	99.90	0.0	99.90	0.0	99.90
10.8	99.42	2.8	99.70	2.9	99.60	2.9	99.75
20.0	99.00	10.8	99.34	6.8	99.07	6.8	99.24
27.7	98.27	15.8	98.71	10.8	99.13	10.8	99.22
33.8	97.91	23.8	98.54	15.8	98.20	15.8	98.55
44.7	96.84	27.7	97.73	20.7	97.50	20.7	95.14
48.7	96.35	37.7	97.00	23.8	97.21	23.8	95.24
51.8	95.94	41.7	96.64	27.7	96.21	27.7	94.89
61.8	94.80	44.7	95.66	30.8	96.02	30.8	91.72
83.6	91.38	51.8	95.08	33.8	94.91	33.8	90.80
99.1	88.50	61.8	93.20	37.8	95.15	37.8	90.26
120.0	85.26	72.8	91.81	41.7	94.06	41.7	86.12
141.0	80.74	83.6	89.97	44.7	93.86	44.7	87.42
...	...	99.1	85.66	48.7	91.11	48.7	82.93
...	...	107.7	83.65	51.8	90.66	51.8	83.32
...	...	120.0	79.99	55.7	89.32	55.7	79.04
...	...	141.0	75.95	61.8	81.46	61.8	70.65
...	...	...	...	72.8	79.51	66.7	64.75
...	...	...	...	83.6	73.09	72.8	60.45
...	...	...	...	91.7	68.66	83.6	55.09
...	...	...	...	107.7	65.50	91.7	53.50
...	...	...	...	114.7	60.16	101.7	41.68
...	...	...	...	120.0	60.03	114.7	38.75
...	...	...	...	132.0	54.98	120.0	36.82
...	...	...	...	...	...	132.0	35.98

80°

181.0 mm.		199.5 mm.		232.5 mm.	
Time in Hr.	Mole % Aspirin	Time in Hr.	Mole % Aspirin	Time in Hr.	Mole % Aspirin
0.0	99.90	0.0	99.90	0.0	99.90
17.0	99.68	17.5	99.39	17.3	99.25
40.8	99.69	40.3	98.16	40.5	98.64
63.3	97.96	62.5	97.41	63.3	97.05
90.5	95.79	90.0	95.24	90.3	94.40
112.5	94.45	111.8	92.39	112.8	86.75
137.0	91.77	136.3	87.80	136.8	84.78
160.5	88.79	160.0	85.69	160.8	77.44
186.5	85.28	186.0	73.86	186.2	72.80
208.8	83.34	208.3	78.40	209.8	67.54
232.8	81.96	232.5	57.31	232.8	60.76
308.8	70.86	308.3	50.20	...	...

tion takes place a film of water will surround each aspirin particle, and the thickness of film will depend on vapor pressure. If monolayer adsorption takes place, each aspirin particle will be partially covered by a layer of water, the extent of coverage being dependent on vapor pressure. (*b*) The water film is rapidly saturated by solution of a portion of the solid aspirin. Decomposition then occurs in solution, and as a molecule of aspirin is removed via hydrolysis it is instantly replaced by one from the solid.

Although only a small portion of the aspirin is

actually in solution, the reaction may be treated as though all the aspirin is in solution and only a small fraction is in active state. This makes it possible to express the concentration in terms of total amount of solid aspirin.

Treatment of this system as a solution makes it possible to use one of the rate constants derived by Edwards (12). The constants of Edwards depending on water concentration are so small as to be negligible, while the concentration of hydroxyl ion in this system is so low that the extent of reaction dependent on it is negligible. Further, since the ionization constant for aspirin is small and ionization would be depressed by hydrogen ion concentration, it is believed that this system involves primarily molecular aspirin. Thus, only one of the constants described by Edwards, k_1, which is associated with hydrogen ion concentration and molecular aspirin, is necessary for the rate expression for this system.

Accordingly the rate equation may be written: $dC/dt = k_1(A)(H^+)$, (Eq. 1), in which C is the concentration of salicylic acid, A is the concentration of aspirin, and H^+ is the concentration of hydrogen ion. Using the definition of the ionization constant for acetic acid, $K = [(H^+)(CH_3COO^-)]/(CH_3COOH)$, (Eq. 2), the concentration of salicylic acid, C, may be substituted for that of acetic acid, since one mole of acetic acid is formed for each mole of salicylic acid, and the decrease in concentration of acetic acid due to ionization is negligible. Also, the concentration of hydrogen ion is equal to that of acetate ion. Thus, the ionization equation may be stated: $K = (H^+)^2/(C)$ (Eq. 3).

In order to convert concentrations to the number of moles contained in volume of solution, V, on the surface of the particles, K, which has units of moles/liter, is multiplied by V to give: $KV = (H^+)^2/(C)$, (Eq. 4). Solving for hydrogen ion concentration, $[H^+] = (KV[C])^{1/2}$, (Eq. 5); and substituting Equation 5 in Equation 1 gives $dC/dt = k_1[A](KV[C])^{1/2}$, (Eq. 6), which applies to any single vapor pressure, and has units of moles $\times V^{-1} \times t^{-1}$. To express the rate in terms of number of moles rather than concentration the equation is multiplied by V, to give $dC/dt = k_1K^{1/2}V^{3/2}[A][C]^{1/2}$, (Eq. 7).

It should be noted that although the units of dC/dt are moles $\times t^{-1}$ those of $[A]$ and $[C]$ are moles $\times V^{-1}$. Since aspirin samples have different weights they are converted to a common basis by expressing both $[A]$ and $[C]$ as per cent of the total number of moles originally present, giving them the units mole% $\times V^{-1}$.

It is possible to express aspirin concentration in terms of salicylic acid concentration, since their sum is always a constant equal to 100. Thus, $[A] + [C] = [A_0] + [C_0] = D_0$, (Eq. 8), and $[A] = D_0 - [C]$, (Eq. 9), where A_0 and C_0 are initial concentrations of aspirin and salicylic acid. This substitution facilitates integration to give $dC/dt = k_1K^{1/2}V^{3/2}(D_0 - [C])\,[C]^{1/2}$, (Eq. 10).

The volume of the solution layer, V, is unknown, but it may be expressed in terms of the Freundlich isotherm equation; thus, for a particular size and shape of particle: $V = k'p^n$, (Eq. 11), in which p is the vapor pressure, k' is the proportionality constant, and n is the order of the sorption reaction with respect to p. Substituting this expression for V into Equation 10: $dC/dt = kp^{3n/2}(D_0 - [C])$ $[C]^{1/2}$, (Eq. 12), where $k = k_1K^{1/2}k'^{3/2}$. By making the substitution $[C]^{1/2} = y$ the following equation is obtained: $2dy/(D_0 - y^2) = kp^{3n/2}\,dt$, (Eq. 13), which can be readily integrated by standard form, giving:

$$\frac{1}{D_0^{1/2}} \ln \frac{(D_0^{1/2} + [C]^{1/2})}{(D_0^{1/2} - C^{1/2})} = kp^{3n/2}t + I \quad \text{(Eq. 14)}$$

From Equation 9 it follows that $(D_0^{1/2} + [C]^{1/2})(D_0^{1/2} - [C]^{1/2}) = [A]$; inserting this into Equation 14:

$$\frac{1}{D_0^{1/2}} \ln \frac{(D_0^{1/2} + [C]^{1/2})^2}{[A]} = kp^{3n/2}t + I \quad \text{(Eq. 15)}$$

Setting t equal to zero, the constant of integration, I, is found to be:

$$\frac{1}{D_0^{1/2}} \ln \frac{(D_0^{1/2} + C_0^{1/2})^2}{A_0} \quad \text{(Eq. 16)}$$

Rearranging and converting to logarithms to the base 10, the final equation is obtained:

$$\log \frac{A_0\,(D_0^{1/2} + C^{1/2})^2}{(D_0^{1/2} + C_0^{1/2})^2 A} = \frac{D_0^{1/2}kp^{3n/2}t}{2.303} \quad \text{(Eq. 17)}$$

If the mechanism proposed is correct, a plot of the left hand side of Equation 17 *versus* time should give a straight line with a slope equal to $(D_0^{1/2} \cdot kp^{3n/2})/2.303$. The data of Table I were plotted in this manner and found to give straight lines. Figure 2 illustrates this type of graph. The values of the slopes of the lines obtained in this manner are shown in Table II.

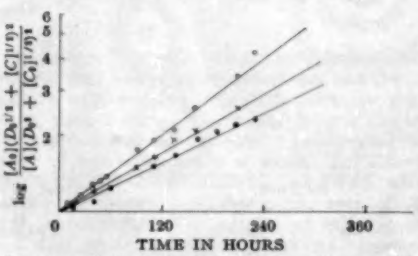

Fig. 2.—Typical plots of decomposition equation (Eq. 17), 80°. Legend: ○—vapor pressure 232.5 mm.; x—vapor pressure 199.5 mm.; ●—vapor pressure 181.0 mm.

It can be seen from the equation of the slope that a plot of log $(D_0^{1/2}kp^{3n/2})/2.303$ *versus* log p should give a straight line with a slope of $3n/2$ and an intercept of log $D_0^{1/2}k/2.303$. These graphs are shown in Fig. 3, and the values of the slopes and intercepts are presented in Table III.

From chemisorption theory (15) n would be expected to be 1, making $3n/2$ equal to 1.5. The results of Table III are in agreement with this within experimental error; thus, the intercepts of Table III were calculated using a value of 1.5 for $3n/2$.

In order to estimate the activation energy for the reaction, k, which is the product of k_1, $k'^{3/2}$ and $K^{1/2}$,

TABLE II.—SLOPES OF THE DECOMPOSITION EQUATION OF ASPIRIN (EQ. 17)

Temp. °C.	Vapor Pressure mm.	Slope = $Dk^{1/3}k_1p^{1/3}/2.303$
80	181.0	1.660×10^{-3} hours^{-1}
	199.5	1.986×10^{-3} hours^{-1}
	232.5	2.475×10^{-3} hours^{-1}
60	68.41	2.772×10^{-3} days^{-1}
	74.50	3.191×10^{-3} days^{-1}
	111.9	5.218×10^{-3} days^{-1}
	120.3	7.547×10^{-3} days^{-1}
50	46.02	7.558×10^{-4} days^{-1}
	54.50	9.980×10^{-4} days^{-1}
	68.30	1.367×10^{-3} days^{-1}
	74.20	1.860×10^{-3} days^{-1}

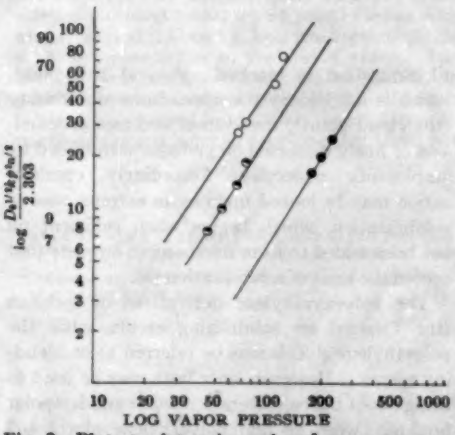

Fig. 3—Plots to determine order of vapor pressure effect. Legend: ○—60°; ◑—50°; ●—80°.

TABLE III

Temp. °C.	3n/2	$Dk^{1/3}k/2.303$
80	1.5	6.953×10^{-7} mm.$^{-3/2}$ hrs.$^{-1}$
60	1.5	4.976×10^{-6} mm.$^{-3/2}$ days^{-1}
50	1.7	2.552×10^{-6} mm.$^{-3/2}$ days^{-1}

TABLE IV. DECOMPOSITION RATE CONSTANTS FOR ASPIRIN AT VARIOUS TEMPERATURES

Temp. °K	$k_1k'^{3/2}$ (Mole %)$^{-1/2}$ Liters$^{1/2}$ Moles$^{-1/2}$ mm.$^{-3/2}$ Hr.$^{-1}$
353.1	44.58×10^{-4}
333.1	12.41×10^{-4}
323.1	6.06×10^{-4}

is divided by $K^{1/2}$, the value of which is known for each of the temperatures studied (16). This leaves $k_1k'^{3/2}$ the values of which are shown in Table IV. From a plot of log $k_1k'^{3/2}$ *versus* $1/T$ the activation energy can be obtained. This value is the sum of two such energies, one associated with the chemical hydrolysis reaction, and the other with the physical sorption reaction. However, since there is no reason to believe that the sorption forces are other than van der Waals, the energy of activation for this reaction can be considered zero (15). Thus the activation energy determined from the slope of an Arrhenius plot is that of the hydrolysis reaction, and was calculated to be 15,065 calories per mole, which is in good agreement with the value of 15,620 determined by Edwards (17).

DISCUSSION

It should be noted that the method used in this work for control of humidity is quite troublesome. The normal variations in temperature of laboratory ovens is sufficient to cause condensation of water on the inside of the desiccators which frequently causes loss of a whole series of samples. Also, data obtained once the per cent aspirin remaining has dropped to a low value, usually around 20–30%, is widely scattered. At this point the samples are commonly observed to become sticky and aggregate or even form a semisolid mass. It is believed that a different physical system exists at this point. Data obtained under such conditions are not reported in this paper and do not follow the mechanism proposed. However, decomposition at this stage is of minor importance.

By use of the equations established in this paper, it is possible to predict the stability of aspirin under known conditions of temperature and humidity. Application of the equation is limited, however, to the particle size used in this work. Further work is in progress in this laboratory to determine the relationship between particle size and rate constant.

It appears that by knowing the ionization constant of a material to be mixed with aspirin, one might use these equations to predict the stability of the mixture. Other factors such as solubility of the added substance will have to be taken into account. Work of this type is planned.

The basic assumptions and treatment of data used in this study show promise of being applicable to many solid medicinals which decompose as a result of adsorption of water.

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The Dispersion of Liquids in Aqueous Solutions of Amphiphilic Compounds*

By WILLIAM J. O'MALLEY,† LUCIANO PENNATI,‡ and ALFRED N. MARTIN

The influence of certain amphiphilic compounds (molecules possessing both hydrophilic and lipophilic character) on the dispersion of nonpolar substances in water is presented in the form of ternary phase diagrams. The dispersion systems studied were peppermint oil-water-Tween 20, peppermint oil-water-polyethylene glycol 400, and benzyl benzoate-water-polyethylene glycol 400. The data were plotted as triangular diagrams to show the effects of the three agents simultaneously. The results suggested that such a method should assist one in the formulation of concentrates for the preparation of aromatic waters and other solubilized products.

NORMALLY INSOLUBLE liquids and solids may be dispersed in aqueous systems by several means, principally among which are solubilization, co-solvency or blending, and emulsification.

These processes are made possible by the presence of an amphiphilic compound, i. e., a substance which has a tendency to a greater or a lesser degree to dissolve in water (hydrophilic character) and in nonpolar solvents (lipophilic or organophilic character). Amphiphilic compounds include solubilizing agents, detergents, emulsifiers, and solvents of a polar-nonpolar nature (1).

McBain (2) defined solubilization as the dispersion of relatively insoluble matter in aqueous solutions of amphiphiles to form thermodynamically stable systems. Above a minimum amount, known as the critical micelle concentration, the amphiphilic compound exists in solution in the form of micelles. Normally insoluble liquids or solids are adsorbed on the surface of the micelles, pass into the micelle interior and dissolve in the nonpolar region, or are adsorbed in the palisade layer of the micelle (3).

Blending and co-solvency are terms used to describe the mutual solubility of normally immiscible liquids in the presence of an amphiphilic agent. Sodium oleate, for example, increases the co-solvency of benzene and propylene glycol, presumably through the formation of bridges between the polar and nonpolar molecules (4).

Emulsification is familiar to every pharmacist; in this discussion it is considered in relation to solubilization. When oil is added to a solution of a solubilizing agent it continues to enter the micelle, which consequently swells until saturation is reached. Beyond this point, which is marked by the appearance of turbidity (the cloud point), the system becomes an emulsion of finely dispersed oil globules surrounded by amphiphilic molecules. Accordingly, emulsification may be looked upon as an extreme case of solubilization, which begins when sufficient oil has been added to form microscopic droplets that exceed the limit of solubilization (3).

The polyoxyethylene derivatives of sorbitan (the Tweens) are solubilizing agents, while the polyethylene glycols may be referred to as blending agents. However, since both may be used to bring about the co-solvency of water and nonpolar liquids, Tween 20 and polyethylene glycol 400 will both be called blending agents in the following discussion.

A number of reports have been published during the last decade on the use of various amphiphilic agents for the dispersion of certain pharmaceutical substances, and they have stressed the advantages of these agents in forming clear, stable solutions of normally insoluble drugs. Monte Bovi (5) used Tween 20 to make a peppermint oil concentrate; Ohmart and Stoklosa (6) discussed some pharmaceutical applications of the Tween and Spans; Nakagawa (7) used the esters of polyoxyethylene to solubilize hormones and vitamins; Loran and Guth (8) studied the use of ethyl alcohol for blending water and castor oil.

The present study was directed toward investigating the dispersion of several nonpolar liquids of pharmaceutical interest in water by means of two amphiphilic agents, namely, Tween 20[1] and polyethylene glycol 400.[2] The effect of dilution and temperature variation was also considered in the Tween 20 system.

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[1] Supplied by the Atlas Powder Company, Wilmington, Del.

[2] Supplied by the Carbide and Carbon Chemicals Company, New York, N. Y.

EXPERIMENTAL

The ternary systems studied were: (*a*) peppermint oil-water-Tween 20, (*b*) peppermint oil-water-polyethylene glycol 400, and (*c*) benzyl benzoate-water-polyethylene glycol 400.

The specific gravities of the ingredients were determined by using a 25-ml. pycnometer at 20°, and the values were found to be as follows: peppermint oil, 0.898; benzyl benzoate, 1.115; Tween 20, 1.102; polyethylene glycol 400, 1.120.

The samples were prepared by pipetting a definite volume of the substance to be solubilized into a 125-ml. flask and adding a definite volume of the solubilizing agent. The solutions were mixed by means of a magnetic stirrer at a slow speed to prevent the formation of air bubbles. The samples were titrated with distilled water until the entire solution remained turbid (cloud point) for one minute as observed in a beam of light passing through the solution perpendicular to the line of vision. The solutions were not allowed to come to equilibrium since this may take several weeks (9, 10). The turbidity, which formed immediately, was taken arbitrarily as the end point, recognizing that it did not represent the equilibrium value but rather supplied a point which could be reproduced upon subsequent tests.

The cloud point was considered as the transition between a solubilized and an emulsified system when the solubilizing agent was used. Although clouding does not necessarily indicate a distinct separation of two phases, it suggests a state of instability, and is referred to later in connection with the phase diagrams as the beginning of a *region of immiscibility*.

The peppermint oil-water-Tween 20 system was studied at temperatures of 10°, 20°, 30°, and 40° using a constant temperature bath to maintain the temperature within ±0.1°. The other systems were investigated at 20°. The volumes were converted to weight using the specific gravities, and the weights of the three ingredients in a given sample were taken to equal 100 per cent.

RESULTS

The results for the system of peppermint oil-water-Tween 20 were plotted as seen in Figs. 1, 2, 3 and 4; and the other systems are shown in Figs. 5 and 6. The volumes of the three ingredients were converted to weight by using specific gravities. Table I gives the weights of the three ingredients necessary to cause a change of phase as is seen in Fig. 1. Regions I/II, II/III and III/IV in Table I represent the limiting values for a visible change of phase from one region to the next. The two curves shown in Fig. 1 represent the per cent by weight and the per cent by volume of the three ingredients.

The Effects of Dilution on the Peppermint Oil-Water-Tween 20 System.—Figure 2 has been used to show some of the difficulties that might accompany the use of surface-active agents to make solubilized concentrates. In this reproduction of a portion of Fig. 1 from zero to 30 per cent peppermint oil, *A* represents a peppermint oil concentrate containing 7.5% oil, 42.5% Tween 20 and 50.0% water, which is diluted with water as needed to yield the aromatic water (5). *A'* was obtained by the dilution of 10.0 ml. of the concentrate to 100 ml. with distilled water, thus forming a solution containing 0.75% peppermint oil and 4.25% Tween 20. The plot indicates that such a concentrate is satisfactory for the preparation of an aromatic water at 25°.

In Fig. 2, concentrate *B*, was used to point up the difficulties that might result from an indiscriminate use of surface-active agents. For example, when 49% peppermint oil, 50% Tween 20 and 1% water were combined a clear solution *B* was produced, but when 10.0 ml. of this solution were diluted with 0.1 ml. of distilled water, a cloudy mixture *B'* resulted. When 1.0 ml. more of distilled water was added,

TABLE I.—THE WEIGHT PER CENT DATA FOR THE SYSTEM OF PEPPERMINT OIL (O), WATER (W), TWEEN 20 (T) AT 20° NECESSARY TO BRING ABOUT A CHANGE FROM ONE PHASE TO THE NEXT

Region I/II			Region II/III			Region III/IV		
O	*T*	*W*	*O*	*T*	*W*	*O*	*T*	*W*
..	..	..	..	..	..	1.5	8.7	89.8
..	..	..	..	..	..	3.2	16.3	80.5
..	..	..	..	..	..	9.0	30.3	60.7
..	..	..	..	..	..	11.5	35.3	53.2
24.5	67.4	8.1	23.7	65.2	11.1	13.6	37.4	49.0
29.3	66.6	6.1	25.4	62.0	12.6	16.4	40.2	43.4
30.4	64.8	4.8	27.8	59.2	13.0	19.5	41.7	38.8
33.9	62.1	4.0	30.8	56.5	12.7	23.2	42.5	34.3
38.2	58.4	3.4	34.5	52.7	12.8	28.2	43.2	28.6
43.8	53.6	2.6	39.9	48.7	11.4	34.8	42.4	22.8
46.4	51.3	2.3	42.3	46.7	11.0	38.0	42.1	19.9
50.3	46.1	3.6	46.3	42.4	11.3	43.8	40.3	15.9
55.9	41.0	3.1	52.6	38.6	8.8	50.4	37.0	12.6
60.0	36.7	3.3	..	..	..	..	..	..
64.7	31.6	3.7	..	..	..	..	..	..
71.8	26.3	2.9	..	..	..	..	..	..
82.5	15.1	2.4	..	..	..	..	..	..

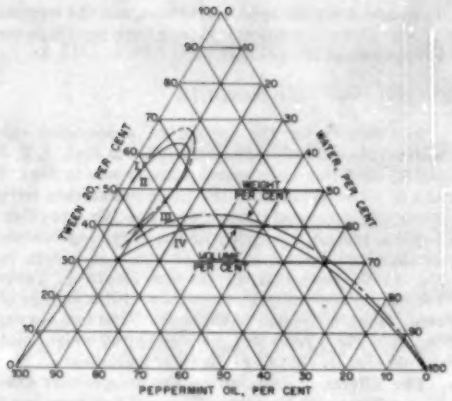

Fig. 1.—The three-component phase diagram showing regions of miscibility and immiscibility at 20°, and comparing weight per cent and volume per cent for peppermint oil, Tween 20 and water.

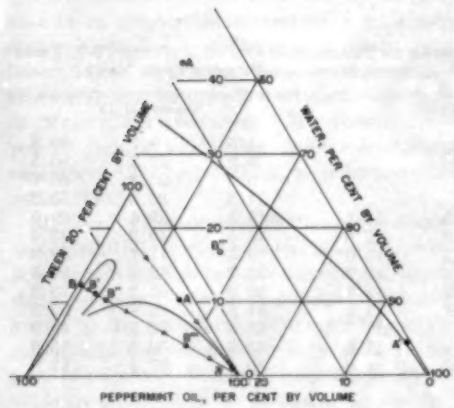

Fig. 2.—Enlargement of a portion of Fig. 1 showing regions of miscibility and immiscibility at 20°. The effects of diluting volatile concentrates for the preparation of aromatic waters are observed by following the lines through points A and B on the insert diagram.

the mixture again became clear as represented by point *B′′*, but when the total volume was brought to 30.0 ml., a turbid mixture *B′′′* was formed, and it remained cloudy until a point was reached where the concentration was approximately equal to the solubility of peppermint oil in water.

In Fig. 3, the grams of oil solubilized were plotted *versus* grams of surface-active agent used. The region plotted covers the same concentrations as Fig. 2 but shows more clearly the solubility of the oil as a function of the amphiphilic agent.

The Influence of Temperature.—Figure 4 depicts the effects of changes in temperature upon the solubility of the peppermint oil-water-Tween 20 mixture. With a decrease in temperature the region of immiscibility below the curve (Region IV) increased while Region II decreased in size. At 10° the mixture was very viscous, and it was not possible to obtain an unambiguous cloud point.

Other Ternary Systems.—Figure 5 shows the phase diagram relationship for peppermint oil-

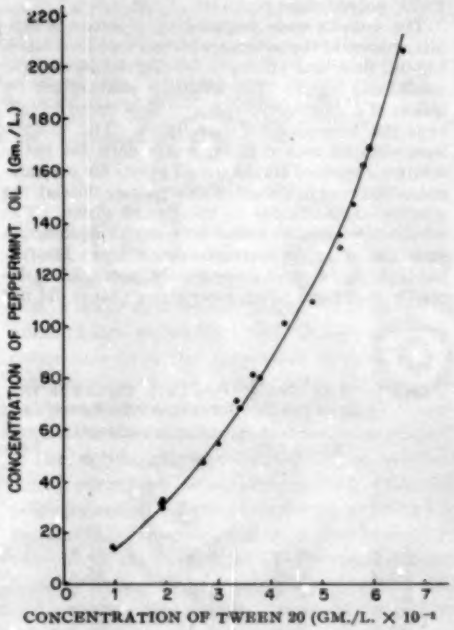

Fig. 3.—The solubilization of peppermint oil with Tween 20 in water.

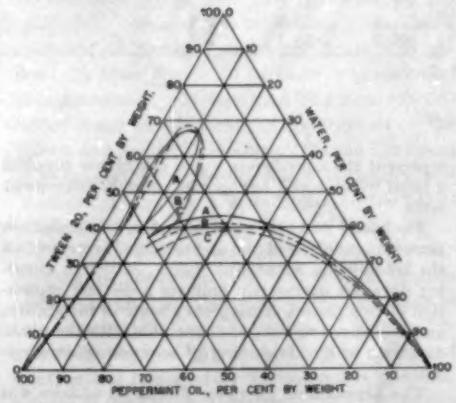

Fig. 4.—Three-component phase diagram showing regions of miscibility and immiscibility at various temperatures for peppermint oil, Tween 20 and water. A, 20°, B, 30°, C, 40°.

water-polyethylene glycol 400, and Fig. 6 illustrates the blending of benzyl benzoate and water by the use of polyethylene glycol 400. The area above the curve in each case represents a clear solution, whereas, the area under the curve is a two-phase region of immiscibility. The differences between the character of the results in Fig. 2 and Figs. 5 and 6 point up the fact that the oil is probably brought into solution by different mechanisms. The solubility shown in Figs. 5 and 6 involves the action of a true co-solvent rather than a micelle-forming surface-active agent.

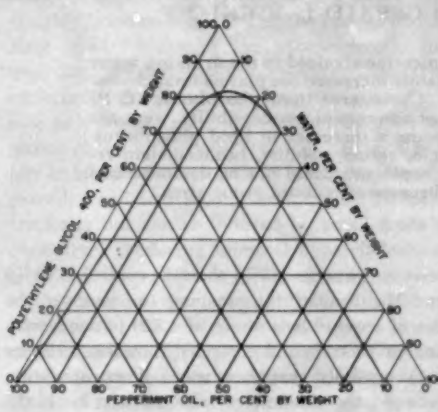

Fig. 5.—Three-component phase diagram showing the regions of miscibility and immiscibility at 20° for peppermint oil, polyethylene glycol 400 and water.

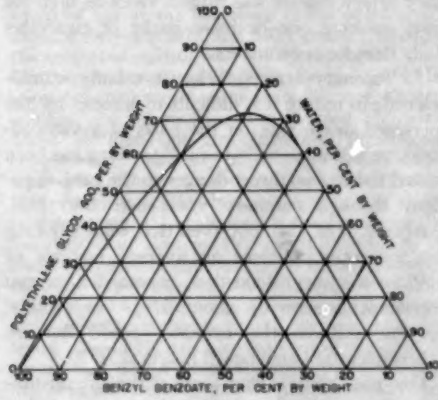

Fig. 6.—Three-component phase diagram showing the regions of miscibility and immiscibility at 20° for benzyl benzoate, polyethylene glycol 400 and water.

SUMMARY AND CONCLUSIONS

Ternary phase diagrams have been used in this study to describe the pharmaceutical systems containing peppermint oil, water and an amphiphilic agent, and they can assist one in the development of solubilized products.

The arrows drawn through the points *A* and *B* in Fig. 2 indicate how the concentrations of the ingredients change upon dilution with water.

Care must be exercised in formulating a solubilized oil concentrate so that a turbid preparation will not result on dilution. The influence of temperature on the mixture was also studied. Moderate changes in temperature have little effect on the phase equilibrium; however, a decrease in ambient temperature may produce turbidity in a normally clear solubilized system.

By comparing Figs. 1 and 5, it can be seen that Tween 20 is more efficient in blending peppermint oil and water than is polyethylene glycol 400. The diagrams also suggest that peppermint oil is probably brought into solution by these two amphiphilic agents by different mechanisms.

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The Effect of Nicotine on Experimental Hypercholesterolemia in the Rabbit*

By DUANE G. WENZEL† and GERALD L. BECKLOFF

The administration of 2.28 mg./Kg./day of nicotine alkaloid in the drinking water of rabbits fed a 0.1% cholesterol diet significantly increased the plasma total cholesterol levels over those of control groups. Cholesterol/lipid phosphorus (C/P) ratios, which may be used as an indication of atherogenic susceptibility, were not raised to the same extent because of concomitant increases in lipid phosphorus. Significant differences in serum ascorbic acid, serum stability, bromsulfalein retention, aortic cholesterol content, and aortic plaques could not be demonstrated at terminal determinations possibly because of reduced group sizes.

THE MEDICAL LITERATURE is replete with investigations concerning the effects of nicotine on the cardiovascular system. Clinically it is generally conceded that nicotine should be avoided in vascular diseases such as thromboangiitis obliterans (1), Raynaud's syndrome (2), peripheral arteriosclerosis (3), and its use is inadvisable in the cardiac sensitivity known as tobacco angina (4). These untoward effects of nicotine are probably related to the decreased peripheral blood flow produced by nicotine as demonstrated by skin temperature fall (5), plethysmographic studies (6), and visual observation of capillary blood flow (7).

While it is apparent that the vasoconstrictive effect of nicotine is deterimental to most of the peripheral vascular diseases, it is difficult to relate its action directly to atherosclerosis. It appears to be more or less tacitly assumed that if nicotine is related to atherosclerosis this relationship is not causal in nature but that its action, at most, only aggravates the already existing disease.

Thienes and Butt (8) treating both rats and rabbits with nicotine found an apparent lack of cardiovascular toxicity as a greater percentage of degenerative vascular changes occurred in the controls than in the experimental group. In a clinical study of 301 male diabetics, however, it was reported 53% of the smokers suffered from atherosclerosis as compared to 23% in the nonsmokers (9). Swiss investigators directed attention to the fact that only 6.7% of a large group of coronary patients were nonsmokers as compared to 25.5% of nonsmokers in a comparable control group (10). They further established that there were more heavy smokers among the atherosclerotic patients (45%) than the control (28.5%) and the heavier the smoking the younger the age of onset. In a study of 1,520 patients with angina pectoris and coronary thrombosis, Sigler (11) concluded that there was a direct correlation between the amount of smoking and both the early appearance of the first coronary occlusion and the occurrence of death due to this cause. Hammond and Horn (12) in a follow-up study of 190,000 men found that the death rate from coronary artery disease was almost twice as high for men smoking one or more packs of cigarettes daily than for nonsmokers.

As coronary artery disease is usually atherosclerotic in nature it is difficult to account for the increased death rate on the basis of a vasoconstrictive action alone. In fact, smoking has been stated to be of no direct danger to the cardiac patient through coronary vasoconstriction (13). Bargeron, *et al.* (14), observed that while smoking one cigarette produces an average increase of 19% in coronary blood flow, myocardial oxygen consumption rises by about 27%. In rabbits, nicotine reduces the coronary blood flow in atherosclerotic Langendorff hearts (15).

One possibility is that some of the circumstantial evidence for the role of dietary fats and cholesterol in the production of atherosclerosis may be related in part to nicotine. For example, it has been observed that a steady increase in coronary artery disease occurred in Norway until 1940, the time of the German occupation. During the occupation when the average daily fat intake was reduced from the usual 159 grams to 71 grams, there was essentially a corresponding decrease in deaths due to circulatory diseases. While the probable role of fats in the changing picture is not denied, it must also be considered that during this same period the tobacco consumption was at a relatively low level (16). A similar possibility exists in studies which have related the low fat intake of certain primitive so-

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cieties to reduction in cardiovascular disease (17). It is likely that here again the low fat diet is accompanied by minimal tobacco consumption.

The following work was designed as a pilot study to explore the possibility of a direct relationship between nicotine and the plasma cholesterol level. Although the atherosclerotic human does not necessarily have hypercholesterolemia, individuals having evidence of coronary disease as a group tend to have higher than normal serum cholesterol levels (18, 19). Experimentally induced atherosclerosis of animals is usually preceded and (or) accompanied by hypercholesterolemia but can be induced by small doses of cholesterol producing minimal hypercholesterolemia (20).

This initial investigation is limited to the examination of the role of nicotine in total plasma cholesterol and lipid phosphorus levels and to the factors which may be responsible for changes observed.

EXPERIMENTAL

Methods.—Male New Zealand white rabbits were used in this study. The animals were six weeks of age at the beginning of the problem and weighed between 2012 and 2333 grams. Group I, the untreated control, was fed Purina Rabbit Chow Checkers and water *ad libitum*. Group II, the cholesterol control, received the same diet except for the addition of 0.1% cholesterol to the feed. This was added by dissolving the cholesterol in sufficient chloroform to uniformly moisten the pellets during thorough mixing. The chloroform was then removed by evaporation in a steam oven. It should be noted that 0.1% cholesterol in the diet is a smaller quantity than that usually administered. A 1% cholesterol diet is ordinarily considered to produce maximal hypercholesterolemia, but the 0.1% level was selected in order to be able to more readily observe possible increases in the plasma cholesterol level. Group III, the nicotine control, received the control diet plus a measured amount of nicotine alkaloid in the drinking water. The amount of nicotine was calculated to supply a quantity of the alkaloid equivalent on a weight basis to the human consumption of two packs of cigarettes daily. The average daily water consumption per rabbit was 350 ml. Four milligrams of nicotine *per os* has been reported to produce the same psychic effects as smoking one cigarette by a habitual smoker (21). Using 70 Kg. as the average adult human weight, the daily two pack rabbit equivalent is approximately 2.28 mg./Kg. of nicotine alkaloid. This quantity was the final daily amount of nicotine in each 350 ml. drinking water of groups II and IV. All drinking containers were plastic. Group IV had the combined treatments of groups II and III; that is, both cholesterol and nicotine.

In order to reduce the possibility of acute nicotine toxicity, the production of tolerance was attempted by gradually increasing the size of the dose. For the first three days the nicotine equivalent of 3.33 "rabbit cigarettes" was given daily. This same quantity was added to the total daily intake at the end of each three-day period until at the end of thirty-six days the full 40 "rabbit cigarette" equivalent was being administered. The quantity of nicotine was adjusted throughout the experiment to correspond to the increase in body weight.

Immediately prior to the initiation of the study, total plasma cholesterol and lipid phosphorus levels were determined for all animals. These were repeated at intervals of four weeks for the twenty-week test period. Total plasma cholesterol was determined by a modification of the Bloor-Sacket method (22). Plasma lipid phosphorus levels were established by a modified Youngberg procedure (23).

At the termination of the test period additional tests were conducted. A serum ascorbic acid analysis was made of each animal according to Lowry (24). The relative serum stability was determined according to a modification of the work of Ressler, *et al.* (25). Using their technique on hypercholesterolemic serum resulted in spectrophotometric readings too turbid to read. Since the results are simply comparative for the control and experimental groups, the following procedure was used which appeared to allow adequate light transmission. Two-tenths milliliter of 0.006 *M* zinc acetate solution was added to 1.5 ml. of serum at thirty-second intervals until a total of 1.8 ml. had been added. Two-tenths milliliter of water was then added and readings were made at 510 mμ with a zero stetting at 70% transmission. Readings were as low as 21% transmission, yet a number reached over 110% and could not be considered as true numbers. A bromsulfalein test for liver function was conducted according to the usual procedure (26).

When the preceding tests were completed the animals were sacrificed and the aortas graded for gross atherosclerotic lesions by the method of Horlick and Katz (27). After grading, the aortas from the left carotid to the right renal artery were dried over potassium hydroxide, extracted as directed by Faber (28) and the cholesterol determined by the same method as used in the plasma cholesterol determinations. Liver and body weights were also recorded.

RESULTS AND DISCUSSION

Table I and Fig. 1 show that the plasma cholesterol levels were approximately equal for all groups at the beginning of the experiment. While the cholesterol levels of the nicotine and control groups then fell slightly and remained relatively constant throughout the twenty weeks of the experiment, both the cholesterol and nicotine-cholesterol groups had an immediate increase in plasma cholesterol at four weeks with leveling at the eight-week test. The nicotine-cholesterol group, however, had a further increase between the eight- and twelve-week periods with subsequent leveling.

The amount of plasma lipid phosphorus was also approximately the same for all four groups at the onset as seen in Table I and Fig. 2, but the changes differed from those of the plasma cholesterol. The nicotine and control group levels showed a falling trend while both the cholesterol and nicotine-cholesterol rabbits had a rise at four weeks with a fall in the levels of the cholesterol group after this test. The lipid phosphorus values of the nicotine-choles-

TABLE I.—MEAN TOTAL CHOLESTEROL, LIPID PHOSPHORUS AND C/P RATIOS OF RABBITS[a]

Group[b]	Time in Weeks 0	4	8	12	16	20
I. Control						
Total Cholesterol ± S.E.	60.2 ± 1.7	38.6 ± 3.4	34.7 ± 2.1	34.1 ± 5.7	29.9 ± 3.6	43.4 ± 5.7
Lipid Phosphorus ±S.E.	6.06 ± 0.33	5.63 ± 0.33	4.78 ± 0.24	2.94 ± 0.30	3.25 ± 0.28	3.44 ± 0.23
C/P Ratio ± S.E.	9.93 ± 0.22	6.86 ± 0.28	7.27 ± 0.29	11.6 ± 1.1	9.21 ± 0.69	12.6 ± 1.3
II. Cholesterol						
Total Cholesterol ± S.E.	50.8 ± 5.5	119.7 ± 20.6	89.1 ± 13.8	97.5 ± 14.6	98.8 ± 18.1	100.4 ± 18.9
Lipid Phosphorus ± S.E.	5.94 ± 0.51	7.59 ± 0.67	6.94 ± 0.73	5.22 ± 0.63	4.22 ± 0.38	4.56 ± 0.40
C/P Ratio ± S. E.	8.56 ± 0.27	15.7 ± 1.2	13.6 ± 0.87	18.7 ± 1.3	23.4 ± 2.3	22.3 ± .18
III. Nicotine						
Total Cholesterol ± S. E.	70.5 ± 5.1	57.3 ± 6.2	47.3 ± 6.7	54.1 ± 6.3	58.2 ± 13.3	47.6 ± 8.1
Lipid Phosphorus ± S. E.	6.41 ± 0.32	5.41 ± 0.21	6.19 ± 0.27	4.75 ± 0.21	5.25 ± 0.59	4.19 ± 0.43
C/P Ratio ± S. E.	11.2 ± 0.74	10.9 ± 1.5	10.8 ± 0.97	12.2 ± 1.1	10.1 ± 0.91	9.29 ± 0.84
IV. Nicotine + Cholesterol						
Total Cholesterol ± S. E.	64.7 ± 3.9	107.2 ± 15.1	112.3 ± 18.1	105.3 ± 31.7	104.7 ± 27.6	103.1 ± 32.8
Lipide Phosphorus ± S. E.	6.34 ± 0.36	7.38 ± 0.65	7.19 ± 0.82	7.13 ± 0.94	7.44 ± 0.60	8.38 ± 1.03
C/P Ratio ± S. E.	10.1 ± 0.32	13.7 ± 0.92	15.8 ± 2.1	26.1 ± 2.3	25.1 ± 2.0	22.1 ± 2.2

[a] Total cholesterol and lipid phosphorus are expressed in mg. %.
[b] Group I, Control, received stock diet; Group II, Cholesterol, 0.1% cholesterol in diet; Group III, Nicotine, 2.28 mg./Kg. nicotine daily in drinking water; and Group IV, Nicotine-Cholesterol, combined treatments of II and III.

terol group remained relatively constant at the eight- and twelve-week periods but began to increase at sixteen weeks.

The total cholesterol/lipid phosphorus (C/P) ratio is thought by some to be a more sensitive index of atherogenic susceptibility than is the plasma cholesterol level alone (29). Table I and Fig. 3 show that the control and nicotine C/P ratios did vary appreciably but that they are raised in both the cholesterol and nicotine-cholesterol groups. While the nicotine-cholesterol combination produced a faster rise in the ratios than cholesterol alone, the ratios of both groups were approximately equal at twenty weeks. Standard errors are included for each group mean in Table I, but because of inherent wide variations in the cholesterol and lipid phosphorus levels of rabbits (30) more sophisticated statistical approaches were employed. The data were first analyzed for variance by the hierachic (31) and orthogonal (32) methods. Since significant differences were obtained between groups in both analyses, the two procedures were combined into a regression comparison analysis (33) to determine if the shape of the curves differ significantly from one another.

The *F* values and their significances are given in Table II. The hierachic analysis of variance reveals significant differences between groups for cholesterol, lipid phosphorus, and the C/P ratios and also between rabbits within groups for cholesterol and lipid phosphorus, but not for the C/P ratios. It may be concluded that there was an overall real difference between the group values although not necessarily for all groups at all times.

The orthogonal analysis of variance also indicates significant differences between the groups and between the periods for the lipid phosphorus and the

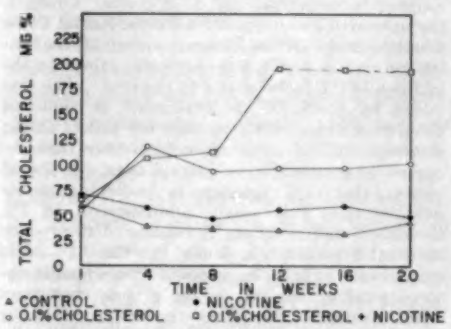

Figure 1.

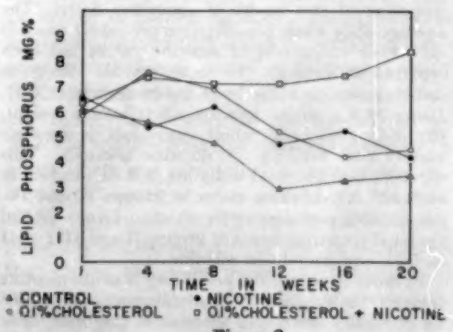

Figure 2.

TABLE II.—ANALYSIS OF VARIANCE

	Hierachic F values	Orthogonal F values
Between groups		
Cholesterol	24.5[a]	73.6[a]
Lipid phosphorus	27.9[a]	28.0[a]
C/P ratio	45.1[a]	88.1[a]
Between rabbits within groups		
Cholesterol	3.16[a]	..
Lipid phosphorus	3.02[a]	..
C/P ratio	1.09[b]	..
Between periods		
Cholesterol	..	1.72[b]
Lipid phosphorus	..	8.19[a]
C/P	..	28.61[a]
Interaction		
Cholesterol	..	5.25[a]
Lipid phosphorus	..	3.92[a]
C/P ratio	..	8.43[a]

[a] $P < 0.001$.
[b] Not significant.

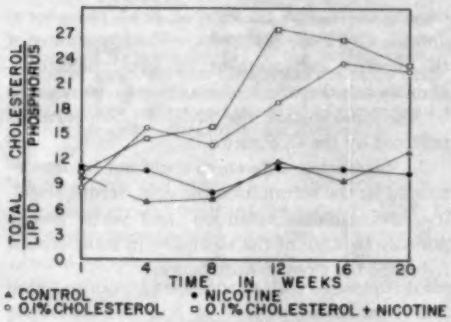

Figure 3.

C/P ratios, but not between the periods for cholesterol. This is not to say that there are not significant differences between periods for cholesterol but only that the study as a whole shows no significant differences. This is clarified by the interaction between periods (P) and rabbits (R) which is significant and demonstrates that real differences do exist between periods but not at all of the times. In other words, as can be seen in Fig. 1, the increases or decreases in plasma cholesterol for all groups did not always proceed in the same direction at one time and there was an overall cancelling effect. Therefore in order to determine whether the nicotine-cholesterol group, for example, had cholesterol values significantly different from those of the control the test period would have to be specified.

Since both the hierachic and orthogonal analyses indicated significant differences between groups, the two procedures were combined into a regression comparison analysis (33) in order to show whether the shapes of the curves differed significantly for the four groups. The results of this analysis are listed in Table III. It can be seen that for several types of curves the differences are significant. This is especially true when they are compared as linear curves.

From the data and its statistical evaluation it may be concluded that while the plasma cholesterol level of the nicotine-cholesterol group was significantly increased by the administration of nicotine over that of the cholesterol group, the lipid phosphorus level of this group also increased. If the C/P ratio is used as an index of atherogenic susceptibility the hypercholesterolemic effect of nicotine is at least partially compensated for by the increase in lipid phosphorus. In order to assess any atherogenic tendency of nicotine it would therefore be necessary to determine the degree of pathological vascular involvement.

The experiment was originally designed to continue for twenty-eight rather than the twenty weeks as shown in the figures and tables. During the last eight weeks a number of animals died in the control, nicotine, and nicotine-cholesterol groups and while values were obtained with the survivors for two more periods they are not included in the data because little reliance could be placed upon the data from small groups. Nine animals were left in the control group, 12 in the cholesterol, five in the nicotine, and four in the nicotine-cholesterol.

At the end of twenty-eight weeks, tests for serum ascorbic acid, serum stability, and bromsulfalein retention were made and the gross aortic lesions, aortic cholesterol, body and liver weights determined

TABLE III.—REGRESSION ANALYSIS OF VARIANCE

	Cholesterol F values				Lipid Phosphorus F values				C/P Ratio F values			
Type of Curve	Control	Cholesterol	Nicotine	Nicotine-Cholesterol	Control	Cholesterol	Nicotine	Nicotine-Cholesterol	Control	Cholesterol	Nicotine	Nicotine-Cholesterol
Linear	18.6[c]	9.38[b]	16.0[c]	52.4[c]	125.5[c]	29.8[c]	26.7[c]	8.13[b]	17.3[c]	209.0[c]	[d]	122.1[c]
Quadratic	19.9[c]	7.53[b]	8.21[b]	7.90[b]	12.8[c]	4.58[b]	[d]	[d]	19.4[c]	24.8[c]	9.74[b]	23.9[c]
Cubic	[d]	8.42[b]	[d]	[d]	12.0[b]	18.4[c]	[d]	[d]	20.2[c]	2.12[d]	1.71[d]	3.21[d]
Quartic	[d]	5.27[a]	[d]	[d]	1.81[d]	[d]	[d]	[d]	15.9[c]	25.3[c]	[d]	[d]
Quintic	[d]	2.48[d]	7.71[a]	3.70[d]	6.11[a]	[d]	[d]	[d]	8.42[a]	20.1[c]	9.85[c]	7.61[b]

[a] <0.05; [b] $P < 0.01$; [c] $P < 0.001$.
[d] Not significant. Where values are not given whole numbers were not obtained.

TABLE IV.—MEAN GROUP VALUES OF TERMINAL DETERMINATIONS

Group	Serum Ascorbic Acid mg. %	Serum Stability, % Transmission	Aortic Total Cholesterol mg. %	Bromsulfalein Retention, %	Body Weight, Gm.	Liver Weight, Gm.
Control	0.73	101	11.1	2.2	3910	111
Cholesterol	0.81	78	16.8	6.7	3490	111
Nicotine	0.76	77	30.6	4.1	3561	113
Nicotine-Cholesterol	0.57	96	18.9	5.6	3435	85

on the remaining animals. These values are listed in Table IV. While they cannot be considered to be meaningful because of the reduced number of animals, they are included for reference purposes. Simple analyses of variance were made for all these determinations but no significant differences could be demonstrated. The body weights are of some interest as the nicotine-cholesterol group ate the least and therefore gained the slowest. Rabbits generally develop more arterial lesions if they are well-fed and thriving than when they gain more slowly (30). In this instance the low weight group had the highest plasma cholesterol level.

These tests were performed in an attempt to determine the mechanism by which nicotine produced hypercholesterolemia. It was thought that the action of nicotine might be related to its effect on ascorbic acid as Bourquin (34) found that the level of ascorbic acid in whole blood was lowered as a result of smoking. This observation coupled with the gross and often complete deficiency of ascorbic acid in the arteries of atherosclerotic humans at autopsy and the fact that scurvy in guinea pigs results in rapidly developing atherosclerosis (35) makes the relationship of potential significance. The role of ascorbic acid in atherosclerosis is possibly a function of its control of the synthesis of cholesterol from active acetate (36) and (or) its importance in general vascular health. Serum stability was studied because of the observation by Ressler (25) that the serum of atherosclerotic individuals was relatively unstable, becoming turbid on the addition of certain metallic salts. Liver function was examined because the liver is the principal organ for cholesterol synthesis, turnover, and excretion. In diseases of the liver various alterations of lipid metabolism occur with associated derrangements of plasma lipid patterns (37).

Gross examination of the aortas revealed plaques in five of the cholesterol group, three of the nicotine group and one of the nicotine-cholesterol group. According to Katz's (27) system for the quantitative evaluation of atherosclerotic lesions they could be graded as class 1 and 2 involvement. This was then minimal atherosclerosis. Once again the inadequate group sizes made comparison inadvisable.

SUMMARY

1. Four groups of twelve rabbits per group were tested five times at four week intervals for total plasma cholesterol and plasma lipid phosphorus. The groups consisted of a control, 0.1% cholesterol diet, 2.28 mg./Kg./day nicotine alkaloid in the drinking water, and combined cholesterol and nicotine. At twenty-eight weeks, studies were made of serum ascorbic acid, serum stability, and bromsulfalein retention. Animals were sacrificed and the aortas were examined for gross lesions and their cholesterol content determined. Terminal body and liver weights were also obtained.

2. Tests of significance demonstrated that the administration of nicotine in addition to a cholesterol-containing diet caused significant increases in plasma cholesterol, lipid phosphorus and the C/P ratio of rabbits. The importance of the nicotine enhancement of hypercholesterolemia as an atherogenic stimulus may be negated by the concomitant rise in lipid phosphorus as reflected by the C/P ratios.

3. Significant differences could not be demonstrated in the serum ascorbic acid, serum stability, bromsulfalein retention, and aortic lesions possibly because of the mortality in some groups following the twenty-week period.

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Effect of the pH of Buffered Paper on the R_f of Alkaloids*

By MICHAEL DEFFNER† and ALICE ISSIDORIDES-DEFFNER

The influence of pH upon the R_f and the nature of the buffer used for the pretreatment of the paper have been investigated for belladonna, strychnos, and opium alkaloids. R_f values at the same pH of the same buffer are correlated to their chemical structure. Variation of R_f with the pH of the buffer, variation of R_f for different buffers of the same pH, the influence of certain anions and cations, molarity of buffer, and the kind of paper used for chromatography are discussed.

ALKALOIDS and other basic substances with a dissociation constant of about 10^{-3} to 10^{-10}, chromatographed on untreated paper with neutral developing solvents, give diffuse elongated spots. This is due to their existence on the paper as ions and as undissociated molecules. These two forms have different R_f values and therefore give elongated spots (1–4).

Strong or very weak bases on the other hand, when chromatographed on untreated paper with neutral solvents, give sharp round spots because they exist in only one form either as ions or as undissociated molecules.

Alkaloids with a dissociation constant between 10^{-3} and 10^{-10} can be chromatographed on paper without "tailing" by addition of an acid to the developing solvent, or by pretreatment of the filter paper with neutral salts or buffer solutions of appropriate pH.

The addition of an acid to the developing solvent generally produces good chromatograms. The dissociation constant of the base determines the amount of the acid to be used. The addition of strong mineral acids or polycarboxylic acids to the developing solvent gives rise to the effect of "demixion" (Entmischung) (1). The ions of the slower moving strong acid remain behind the solvent front forming a second front, the front of "demixion." If the alkaloids appear above or below the front of "demixion," this effect does not interfere in most cases with the identification and separation of the substances. However, if an alkaloid appears in the vicinity of the front of "demixion," double spots are formed which make the measurement of the R_f and separation of a mixture difficult. By increasing or decreasing the concentration of the acid, and thus moving the front of "demixion" above or below the spot of the alkaloid, the formation of double spots can be prevented.

The use of filter paper pretreated with neutral salts or buffer solutions also prevents "tailing." In the first case, the acid of the anion of the salt is added to the developing solvent; whereas in the second case, neutral developing solvents are used.

The R_f of an alkaloid chromatographed on paper pretreated with buffer solutions with *n*-butyl alcohol or isobutyl alcohol saturated with the same buffer solution as the developing solvent, depends on the pH and the nature of the buffer used (5). The lower the pH, the lower the R_f value of the alkaloid will be since the ion has lower R_f value than the undissociated base. Thus the R_f increases with increasing pH and becomes constant at a higher pH.

The purpose of this investigation was to determine the variation of the R_f for various alkaloids with different buffers. The alkaloids investigated were apoatropine, atropine, hyoscyamine, scopolamine, brucine, strychnine, codeine, morphine, papaverine, and 1-benzyl-3-ethyl-6,7-dimethoxy-isoquinoline (isaverine, Merck).

EXPERIMENTAL

Apparatus and Reagents.—Beckman model G pH meter, *n*-Butyl alcohol reagent grade, chloroform

* Received January 8, 1957 from the Vitarine Greece Co., Athens, Greece.
† Associate Professor, University of Athens, Athens, Greece. The authors wish to thank Merck AG. Darmstadt, and The Vitarine Co., Inc., New York, for supplying the compounds used in this study.

reagent grade, McIlvaine's buffer (6), citrate buffer (7), phosphate buffer 0.2 *M* U. S. P. XV, tartrate buffer 0.2 *M* (7), acetate buffer 0.2 *M* (7), lactate buffer 0.2 *M* (7), phthalate buffer U. S. P. XV, and Dragendorff's reagent as modified by Munier and Macheboeuf (1).

Preparation of Papers.—Strips of Whatman No. 2 paper, 4 x 30 cm., were immersed in different buffer solutions, air dried, sewn lengthwise with a white cotton thread in order of ascending pH, formed to fit the glass cylinder, and then placed therein.

Chromatography.—Glass cylinders (13 cm. diam., 45 cm. height) were used. The substances were applied with a micropipet as free bases in a chloroform solution (20 mcg. each time). The ascending method was used with *n*-butyl alcohol saturated with water as the developing solvent. A small beaker containing the water phase was placed at the bottom of the chromatographic jar, the hollow paper form was placed therein, and left six to fifteen hours for equilibration. The developing solvent was then added and the chromatograms were developed in the dark for six to eight hours (height: 20–25 cm.). The chromatograms were air dried and sprayed with Dragendorff's reagent and the R_f values were calculated, measuring the distance from the point of application to the leading edge of the spot. All chromatograms were run in duplicate and the R_f values, although varying with temperature, did not differ by more than 7%.

The pH of the buffer solutions was measured before and after immersion of the filter paper and after shaking the buffer solution with *n*-butyl alcohol. As Table I shows for citrate buffer, the pH remains unchanged after immersion of the paper, but increases slightly after shaking with *n*-butyl alcohol.

TABLE I.—pH OF BUFFER AFTER IMMERSION OF PAPER AND AFTER SHAKING WITH *n*-BUTYL ALCOHOL

Initial pH	pH After Immersion	pH After Shaking With *n*-Butyl Alcohol
1.22	1.22	1.32
2.20	2.15	2.30
3.20	3.20	3.30
4.30	4.30	4.40
5.40	5.40	5.50
6.00	5.95	6.10

Use of Whatman Nos. 1 and 4 and Schleicher and Schüll No. 2043b paper gave slightly different R_f values, but showed the same variation of R_f with the pH of the buffer solution used for the pretreatment of the paper. R_f values were always lower on Whatman No. 1 and Schleicher and Schüll No. 2043b than those obtained on Whatman No. 2 paper. On Whatman No. 4 paper they were highest. Whatman No. 1 paper sometimes gave "tailing," whereas Whatman No. 4 paper gave more diffuse spots. Changes in the molarity of the buffer did not bring about any appreciable changes in the R_f values, but the lower the molarity, the more elongated were the spots. The use of citrate buffer, prepared from secondary sodium citrate with the addition of HCl and NaOH, respectively (7), gave different R_f vs. pH graphs from those obtained when using citrate buffer prepared from citric acid by adding tertiary sodium citrate. Figure 1 shows that the R_f values up to about a pH of 5.0 are higher for the first buffer than those for the second. This is due to the influence of chlorine ions of HCl used for the preparation of the first buffer.

With pH's above 5.0 there is no influence of chlorine ions because NaOH instead of HCl was added to the buffer. The use of KOH instead of NaOH in the buffer gave higher R_f values which indicated some influence of the cations on the R_f values of the alkaloids. Figures 2, 3, 4, and 5 illustrate the dependency of the R_f values of the alkaloids on the pH for: citrate, McIlvaine's, phosphate, and tartrate buffers. The R_f values of the alkaloids at different pH's for acetate and lactate buffers are shown in Tables II and III. The R_f values of all alkaloids increased steadily with increasing pH for those two buffers. The R_f values for phthalate buffer are not shown because they caused only slight variation.

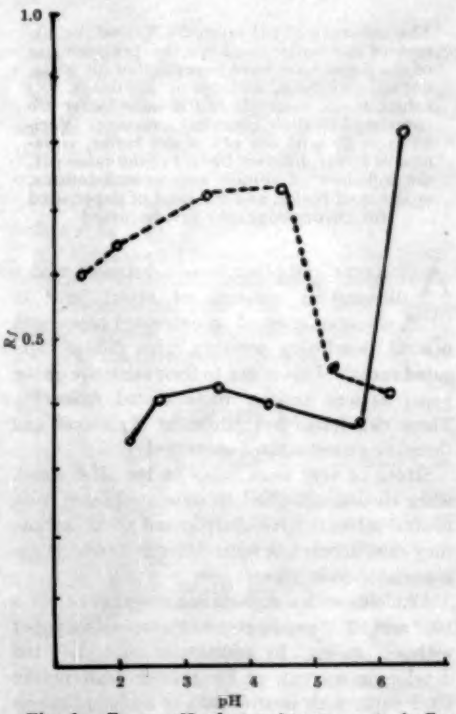

Fig. 1.—R_f vs. pH of atropine, O- - -O, buffer prepared from secondary sodium citrate with the addition of HCl and NaOH; O——O, buffer prepared from citric acid and tertiary sodium citrate.

DISCUSSION

The R_f values of the compounds studied depended primarily on the partition between the stationary

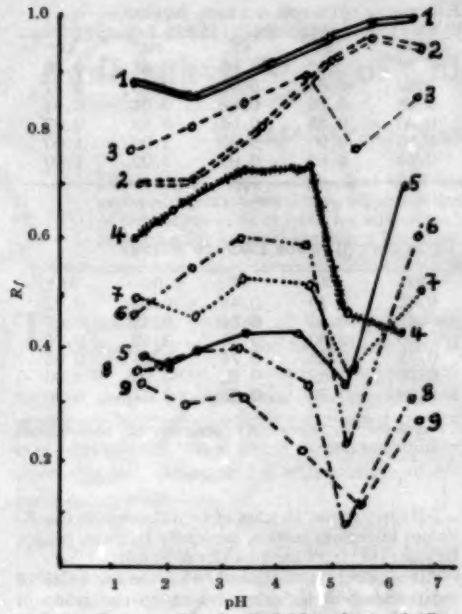

Fig. 2.—R_f vs. pH of alkaloids, citrate buffer.

See Fig. 5 for legend of curves

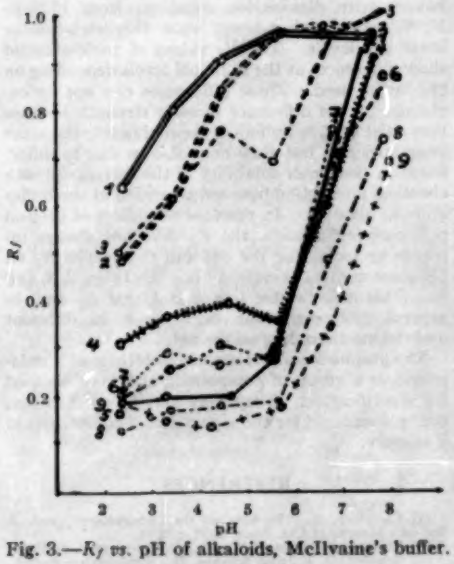

Fig. 3.—R_f vs. pH of alkaloids, McIlvaine's buffer.

See Fig. 5 for legend of curves

phase—adsorbed water—and the moving phase—organic solvent. The highly lipophilic compounds travelled as fast as the organic solvent having high R_f values. The hydrophilic ones having low R_f values remained close to the starting line. Thus the larger the alkyl group, the higher the R_f. Isoalkyl

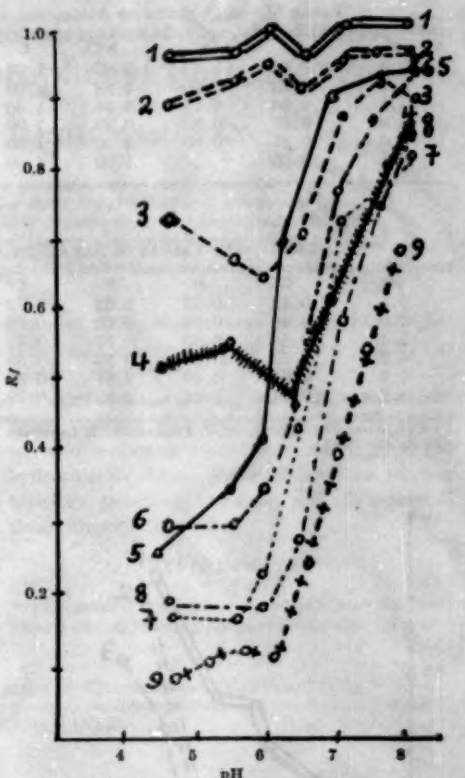

Fig. 4.—R_f vs. pH of alkaloids, phosphate buffer.

See Fig. 5 for legend of curves

compounds had lower R_f values than their *n*-alkyl analogs. Hydroxy compounds also had lower R_f values than their alkyl analogs. Cations and anions gave lower R_f values than the undissociated molecules, bases, and acids (8).

In the group of belladonna alkaloids, apoatropine has atropic instead of tropic acid in the molecule, the CH—CH_2OH group being substituted by a C=CH_2 group. Apoatropine has therefore higher R_f values than atropine at all pH's of all buffers used. Scopolamine has, instead of the tropanol nucleus, an oscine nucleus with an oxygen bridge. Scopolamine has, at pH's above 5.50 and in acetate buffer, higher R_f values than atropine, but in all other buffers below pH 5.50, lower R_f values than atropine; probably because of the formation of oxonium ions at the lower pH levels.

In the group of strychnos alkaloids, brucine, a dimethoxystrychnine, has lower R_f values than strychnine.

The opium alkaloid codeine, is more lipophilic than morphine and therefore has higher R_f values. In the group of opium alkaloids with an isoquinoline nucleus, papaverine, because of the two additional —OCH_3 groups and the lack of the —C_2H_5 group, has lower R_f values than isaverine (1-benzyl-3-ethyl-6,7-dimethoxy-isoquinoline).

TABLE II.—R_f VALUES OF ALKALOIDS AT DIFFERENT pH'S FOR ACETATE BUFFER

pH	C	M	P	I	ST	B	AT	SC	AP[a]
3.3	0.84	0.64	0.86	1.00	0.74	0.60	0.60	0.84	0.76
3.7	0.89	0.70	0.88	1.00	0.80	0.64	0.74	0.90	0.87
4.3	0.94	0.72	0.91	1.00	0.88	0.76	0.82	0.94	0.94
4.9	0.97	0.82	0.92	1.00	0.95	0.85	0.94	0.99	0.97
5.6	0.97	0.80	1.00	1.00	0.95	0.90	0.96	1.00	1.00
6.1	0.95	..	1.00	1.00	0.95	0.86	0.98	1.00	1.00

TABLE III.—R_f VALUES OF ALKALOIDS AT DIFFERENT pH'S FOR LACTATE BUFFER

pH	C	M	P	I	ST	B	AT	SC	AP[a]
2.5	0.42	0.32	0.63	0.72	0.42	0.40	0.48	0.32	0.62
3.0	0.46	0.39	0.66	0.76	0.48	0.42	0.54	0.38	0.67
3.4	0.54	0.39	0.72	0.82	0.60	0.50	0.60	0.46	0.76
4.2	0.62	0.45	0.82	0.85	0.72	0.62	0.74	0.56	0.86
4.8	0.64	0.40	0.84	0.86	0.76	0.68	0.82	0.66	0.90
5.2	0.64	0.44	0.84	0.78	0.78	0.70	0.82	0.67	0.90

[a] C, Codeine; M, Morphine; P, Papaverine; I, Isaverine; ST, Strychnine; B, Brucine; AT, Atropine; SC, Scopolamine; AP, Apoatropine.

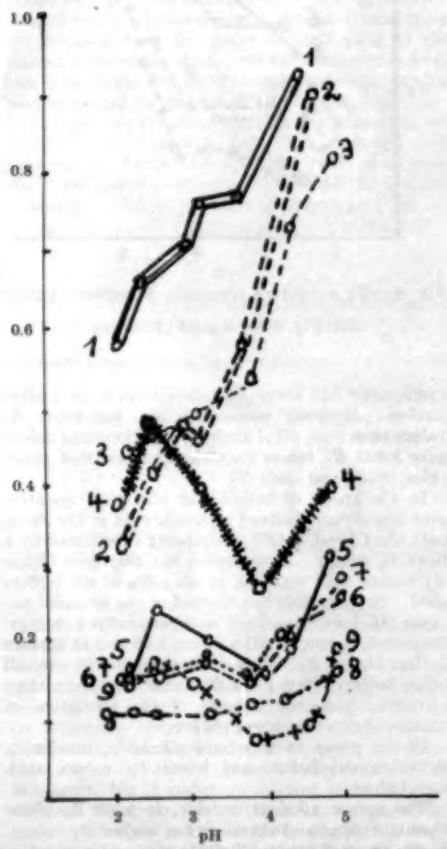

Fig. 5.—R_f vs. pH of alkaloids, tartrate buffer. Figures 2, 3, 4, and 5: 1---1 isaverine, 2---2 papaverine, 3---3 apoatropine, 4---4 atropine, 5---5 scopolamine, 6---6 strychnine, 7---7 codeine, 8---8 brucine, 9---9 morphine.

l-Hyoscyamine, in spite of its difference in the R_f values in certain buffers, especially tartrate, cannot be separated from atropine, its *dl*-isomer.

All alkaloids investigated, with the exception of papaverine and isaverine, belong to the group of alkaloids with dissociation constants from 10^{-3} to 10^{-7} and show a variation of R_f with pH, the R_f generally increasing with increasing pH. Papaverine and isaverine, which belong to the group of alkaloids with dissociation constants from 10^{-7} to 10^{-10}, being weaker bases, show this variation at lower pH levels. The R_f values of each alkaloid show differences at the same pH levels depending on the buffer used. These differences can not be explained by the difference in ionic strength because they exist even in buffers of approximately the same ionic strength, but they may also be due to differences in the water solubility of the solvent (9) or a chemical interaction between the anion of the buffer and the alkaloid. In the case of buffers of di- and poly-carboxylic acids, the R_f does not always increase by increasing the pH and the graphs R_f *vs.* pH show minima at certain pH levels (Figs. 2, 3, and 5). This may be due to the fact that di- and, in general, poly-carboxylic acids exist in different ionic forms depending on the pH.

The graphs R_f *vs.* pH are characteristic of a compound or a group of compounds and may be used for identification. They may also be used to find the optimum pH for the separation of compounds in a mixture.

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The Synthesis and Pharmacology of N-Substituted Aminoalkyl Esters of Chlorophenyl Carbamic Acid*

By ELIAS EPSTEIN and DANIEL KAMINSKY

Dialkylamino and morpholino alkyl esters of *ortho*, *meta*, and *para*-chlorophenyl carbamic acids were prepared and tested for their local anesthetic efficiency (ratio of potency to toxicity) and for irritation. Most of these compounds proved to be too irritating for clinical use but a few were sufficiently low in irritating properties to warrant further investigation.

THE GREATEST NUMBER of local anesthetics introduced for use by the medical and dental professions are esters of benzoic or substituted benzoic acids. Nevertheless, the property of producing local anesthesia is not limited to this type of structure, but is found in amides, amino-alcohol ethers, esters, and a wide variety of organic compounds.

Several of the esters of phenylcarbamic acids have been prepared and their anesthetic action noted (1–19). It was of interest to us to prepare the N-substituted aminoethyl and aminoisopropyl esters of the three isomeric chlorophenyl carbamates and to screen them for their relative anesthetic efficiency (ratio of relative potency to relative toxicity).

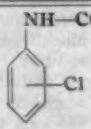
Table I lists the melting points of the anesthetic bases together with the melting points, analyses, and molecular weight determinations of the hydrochloride salts. Table II lists the relative toxicities, potencies, irritation, and efficiencies of these compounds.

EXPERIMENTAL

The general method of synthesis was the well-known one of reacting an isocyanate with an active hydrogen, in this case the hydrogen bonded to the oxygen atom of the aminoalcohol. The chlorophenyl isocyanates react with the aminoalcohols in an inert solvent such as benzene to form the carba-

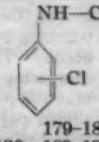
TABLE I.—N-SUBSTITUTED AMINOALKYL ESTERS OF CHLOROPHENYL CARBAMIC ACID

(*a*)



No.	Cl Position	R	m. p. base	m. p. HCl	Formula	Per Cent Ionic Cl Theory	Per Cent Ionic Cl Found	Molecular Weight Theory	Molecular Weight Found
1	*o*	Dimethylamino	Oil	166–168	$C_{11}H_{14}O_2N_2Cl_2$	12.70	12.64	279	274
2	*o*	Diethylamino	Oil	160–163	$C_{13}H_{20}O_2N_2Cl_2$	11.55	11.49	307	305
3	*o*	Morpholino	73–75	193–196	$C_{13}H_{16}O_3N_2Cl_2$	11.04	10.91	321	319
4	*o*	Di-isopropylamino	Oil	136–141	$C_{15}H_{24}O_2N_2Cl_2$	10.58	10.44	335	331
5	*o*	Dibutylamino	Oil	138–145	$C_{17}H_{28}O_2N_2Cl_2$	9.76	9.58	363	359
6	*m*	Dimethylamino	74–77	169–171	$C_{11}H_{14}O_2N_2Cl_2$	12.70	12.49	279	273
7	*m*	Diethylamino	Oil	140–144	$C_{13}H_{20}O_2N_2Cl_2$	11.55	11.38	307	305
8	*m*	Morpholino	119–121	222–224	$C_{13}H_{16}O_3N_2Cl_2$	11.04	10.97	321	320
9	*m*	Di-isopropylamino	Oil	164–167	$C_{15}H_{24}O_2N_2Cl_2$	10.58	10.42	335	332
10	*m*	Dibutylamino	Oil	168–173	$C_{17}H_{28}O_2N_2Cl_2$	9.76	9.98	363	366
11	*p*	Dimethylamino	56–58	173–176	$C_{11}H_{14}O_2N_2Cl_2$	12.70	12.89	279	280
12	*p*	Diethylamino	Oil	175–177	$C_{13}H_{20}O_2N_2Cl_2$	11.55	11.29	307	306
13	*p*	Morpholino	166–169	229–230	$C_{13}H_{16}O_3N_2Cl_2$	11.04	10.89	321	318
14	*p*	Di-isopropylamino	Oil	181–183	$C_{15}H_{24}O_2N_2Cl_2$	10.58	10.43	335	331
15	*p*	Dibutylamino	Oil	160–161	$C_{17}H_{28}O_2N_2Cl_2$	9.76	9.91	363	364

(*b*)



16	*o*	Dimethylamino	Oil	179–182	$C_{12}H_{16}O_2N_2Cl_2$	12.10	11.99	293	292
17	*p*	Dimethylamino	124–126	189–191	$C_{12}H_{16}O_2N_2Cl_2$	12.10	11.98	293	290

* Received September 23, 1937, from the Research Laboratories of the Novocol Chemical Mfg. Co., Inc., Brooklyn, N. Y.

We are indebted to Ann Hartman for assistance in the pharmacological testing of these compounds.

TABLE II.—PHARMACOLOGY OF N-SUBSTITUTED AMINOALKYL ESTERS OF CHLOROPHENYL CARBAMIC ACIDS

No.	Relative Potency: Rabbit Eyes[a]	Relative Potency: Guinea Pig Wheal[b]	Relative Potency: Guinea Pig Sciatic Nerve[b] No Epin.	Relative Potency: Guinea Pig Sciatic Nerve[b] With Epin.	Relative Toxicity: White Mouse[b] Intraperitoneal	Relative Toxicity: White Mouse[b] Subcutaneous	Relative Toxicity: Rabbit Intravenous	Irritation: Eye	Irritation: Skin	Relative Efficiency[b]
1	0.5	0.2	1.7	1.2	1.1	0.9	3	0	0	0.1
2	0.3	3.7	5	1.1	1.5	2	>4	0	0	2.5
3	0.3	0.08	0.3	0.8	0.8	0.8	0.7	++	+	0.1
4	1	1.1	2	3	3	2	>4	0	+	0.4
5	2	2.4	1.5	4	0.5	0.5	>4	++	+	4.8
6	[e]	0.9	1.6	2	0.7	0.8	0.8	++	+	1.3
7	1	1.5	1.4	2	1.1	2	>4	++	+	1.4
8	0.2	0.5	1.0	0.5	0.5	0.6	<0.6	+	+	1.0
9	1	1.5	3	4	1.5	3	>4	+	+	1.0
10	2	4.1	1.5	3	0.9	0.5		+	+	4.5
11	[e]	0.6	0.9	1.7	1.6	3		++	++	0.4
12	0.2	2.1	1.3	1.7	0.9	1.0	1.6	+	0	2.3
13	0.2	0.2	0.3	<0.1	0.5	0.7	0.7	0	+	0.4
14	0.3	0.4	2	1.1	0.6	1.0	1.3	0	0	0.7
15	4	2.4	[d]	[d]	[d]	[d]	[d]	0[e]	[d]	
16	1	1.1	4	3	0.8	0.8	1.1	+	+	1.4
17	[e]	1.1	1.7	0.8	0.7	0.9		++	++	1.6

[a] Cocaine equals 1. [b] Procaine equals 1. [c] Too irritating for test. [d] Too insoluble for test. [e] No apparent irritation at 0.2%.

mates in good yield. Several of the hydrochlorides were insoluble in water and were isolated by adding dilute hydrochloric acid to the benzene reaction mixture and filtering. The anesthetic compounds were purified by recrystallization of the hydrochloride salts from isopropyl alcohol.

The *ortho*, *meta*, and *para*-chlorophenyl isocyanates were commercially available. The aminoalcohols, also commercially available, were carefully fractionated before use.

Diethylaminoethyl Ester of *m*-Chlorophenyl Carbamic Acid.—To a solution of 16.9 Gm. (0.11 mole) *m*-chlorophenyl isocyanate in 50 ml. of dry benzene was added 11.7 Gm. (0.1 mole) of diethylaminoethanol dissolved in 50 ml. of dry benzene. The mixture was allowed to stand at room temperature for twenty-four hours and then heated to the boiling point. After cooling to room temperature, the mixture was filtered and the filtrate extracted with two 250-ml. portions of 15% hydrochloric acid. The acid extract was made alkaline with excess concentrated ammonium hydroxide. The anesthetic base was extracted with two 200-ml. portions of ether, dried over anhydrous sodium sulfate, and bone-charred. The ether solution was acidified with anhydrous hydrochloric acid. The precipitate on recrystallization from 99% isopropyl alcohol yielded 29.3 Gm. (96% of theory) diethylaminoethyl ester of *m*-chlorophenyl carbamic acid, as white crystals, m. p. 140–142°.

PHARMACOLOGY

The anesthetic potency of these compounds was determined by three methods: topical application to the rabbit cornea; blocking the sciatic nerve of the intact guinea pig; and by wheal tests on the back of the guinea pig. The toxicity of these compounds was determined subcutaneously and intraperitoneally on white mice and intravenously on rabbits.

Topical Anesthetic Potency.—This method, originated by Koller and described by Hirschfelder and Bieter (20), was modified as follows: 0.25 ml. of solution was instilled into the conjunctival sac of the rabbit's eye and the lower lid was slightly raised to allow the solution to bathe the cornea of the eye for thirty seconds. The presence of anesthesia was determined by the absence of the wink reflex when the cornea of the eye was stimulated by a long hair obtained from the rabbit's maxilla. The relative topical anesthetic potency was determined by extrapolating results at different concentrations to the concentration which would give a duration of local anesthesia equal to a 1% cocaine hydrochloride solution.

Conductive Anesthetic Potency.—This method, originally introduced by Shackell (21), involves blocking the sciatic nerve in the guinea pig under conditions not too different from those in clinical practice. Two-tenths of a ml. of solution was injected into the bony furrow between the trochanter of the femur and the vertebral column where the sciatic nerve emerges from the spinal column. The loss of sensation at the calf of the leg was determined by pinching lightly with a tweezer. The relative potency was determined by extrapolating the concentration which gave anesthesia for the same duration as one per cent procaine solution. This was repeated, using solutions containing 1:100,000 epinephrine.

Infiltration Anesthetic Potency.—The method used consisted of raising wheals on the guinea pig back with 0.1 ml. of different concentrations of the compound, waiting fifteen minutes, and then determining the presence or absence of anesthesia by touching the center of the wheal with the point of a needle. The relative potency was determined by comparing the lowest concentration of the anesthetic that will produce anesthesia 50% of the time on the guinea pig and comparing this value to that obtained with procaine hydrochloride.

Subcutaneous Toxicity on White Mice.—The relative subcutaneous toxicity of each compound was determined by comparing the LD_{50} with that obtained for procaine hydrochloride. The tests were run at three critical dose levels, with three animals at each level, using a minimum of nine

animals per compound. The critical dose is one near or at the LD_{50}. The numerical value of the LD_{50} was calculated by the method of summation of the animals living and dying at each dose level and determining the point where the two curves would cross (22, 23). White male Swiss mice of approximately 15 Gm. in weight were carefully injected subcutaneously with a 27 gauge needle over the abdominal area with a 2–4% solution of the compound. If no wheal formed or if leakage was observed during or just after the injection, the results of that test were discarded. Toxic symptoms developed in a few minutes and lasted up to several hours. The animals were observed for a twenty-four hour period and those animals alive and appearing normal were considered to have survived the test.

Intraperitoneal Toxicity on White Mice.—The relative intraperitoneal toxicities were determined by comparing the LD_{50} of these compounds with that of procaine hydrochloride in the same manner as for the subcutaneous injection. Toxic symptoms developed sooner and lasted for a shorter time than with the subcutaneous injection. The same number of animals and dose levels were used as for the determination of the subcutaneous toxicity.

Intravenous Toxicity on Rabbits.—The relative intravenous toxicity on rabbits was determined for the most part only on those compounds which were not too irritating. A minimum of four rabbits per compound was used in this determination. The intravenous dose was diluted with normal saline solution to a volume equal to 2 ml. per Kg. of body weight and injected over a period of one minute into the marginal ear vein of the rabbit. Toxic symptoms usually developed immediately after the injection was completed. Those animals alive after twenty-four hours were considered to have survived.

Irritation Test on the Rabbit Eye.—The eye of the rabbit was closely examined for evidence of irritation, one hour and twenty-four hours after instillation of a 1% solution of the compound. The results are recorded in Table II as follows: (*a*) Little or no irritation: 0, no or slight hyperemia of the conjunctiva. (*b*) Moderate irritation: +, moderate hyperemia of the conjunctiva with the eyelids remaining open and no exudate present. (*c*) Severe irritation: ++, severe hyperemia of the conjunctiva with the eyelids closed with or without the presence of exudate.

Trypan Blue Irritation Test.—All of the compounds were tested for irritation on the rabbit skin. A minimum of two rabbits were used for each compound. The method consisted of raising a wheal on the abdomen of the rabbit and subsequently injecting a trypan blue solution intravenously in the ear. The method is essentially that of Weatherby (24). The results were recorded in Table II, using the same classification as for the corneal irritation test.

Relative Anesthetic Efficiencies.—Relative anesthetic efficiencies were determined by dividing the relative potency, as determined via the guinea pig wheal test, by the relative intraperitoneal toxicities on white mice. The guinea pig wheal test for anesthetic potency of commercially used anesthetics gives a close correlation to clinical findings. The relative toxicity obtained with intraperitoneal injection on white mice was used, since it represents a value between that of the intravenous toxicity, where the drug is absorbed rapidly; and that of the subcutaneous toxicity, where the drug is absorbed slowly.

DISCUSSION AND SUMMARY

Seventeen N-substituted aminoalkyl esters of chlorophenyl carbamic acid were prepared and tested pharmacologically. The relative potency, toxicity, irritation, and anesthetic efficiency were determined. The greater number of this group of compounds were irritating. The toxicities were in the same order of magnitude as other commonly used anesthetics.

The following general correlation between molecular structure and anesthetic efficiency can be made.

1. The diethyl and dibutyl aminoethyl esters are more efficient than the dimethyl, diisopropyl, and morpholino aminoethyl esters.

2. N-Substituted aminoisopropyl esters are more efficient than the corresponding N-substituted aminoethyl esters.

3. The position of the chlorine atom on the benzene ring does not impart any clear cut differences in effectiveness of the compound.

Several of the more promising anesthetic compounds are being tested clinically.

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Determination of the Minimal Carcinogenic Dose$_{50}$ of Methylcholanthrene on Mouse Epidermis*

By RONALD F. GAUTIERI and DAVID E. MANN, Jr.

The minimal carcinogenic dose$_{50}$ (MCD_{50}) of methylcholanthrene necessary to induce squamous cell carcinomas on the epidermis of CF-1 mice was found to be 504 micrograms applied through 21 biweekly applications of 0.02 milliliter of a 0.12 per cent solution of methylcholanthrene. The MCD_{50} was confirmed by a second experiment which yielded similar results.

SINCE TANNENBAUM and Silverstone (1) have conclusively demonstrated that the effect of an inhibitory procedure on tumor incidence with moderate doses of a carcinogen was obliterated by massive doses of the same agent, it becomes imperative to define the minimal dose of a carcinogen that is necessary to induce growths in a given percentage of animals in a definite time interval. Methylcholanthrene has been shown to be both consistent and potent in its ability to produce epidermal tumors when painted on the backs of mice (2, 3, 4). Similarly, this agent is valuable, when used as the strong carcinogen, in studying additive and inhibitory effects in conjunction with weaker carcinogens (2, 3, 4). If a satisfactory standardized dose/response relationship could be established for this agent, experimental procedures involving the inhibition or the enhancement of tumor growth would be more clearly evaluated with a better understanding of the mechanisms underlying the cancerization process.

Cramer and Stowell (5) have devised a technique that involves a quantitative estimation of the amount of methylcholanthrene painted on the interscapular region of mice with each stroke of a number 4 camel's hair brush. Berenblum and Schoental (6) have stated that if this carcinogen is applied for a considerable length of time and the effective concentration on the epidermis is sufficient, methylcholanthrene will induce cancers in 100 per cent of the mice exposed to it. The injectable tumor dose$_{50}$ of methylcholanthrene has been found to be 0.021 mg. by Bryan and Shimkin (7). Berenblum (8) has estimated that the topical tumor doses of various carcinogens appear to be approximately 50 times greater than the corresponding injectable doses.

It is the purpose of this paper to define a MCD_{50} for methylcholanthrene on mouse epidermis, utilizing various concentrations of this carcinogen and employing newer techniques for the controlled administration to the sheared, interscapular region of albino mice.

MATERIALS AND METHODS

The experimental plan was conducted in two separate parts for the purpose of evaluating the effectiveness and the reproducibility of the procedures in producing dose/response relationships that are relatively constant with the concomitant verification of the MCD_{50}.

In part I, 120 CF-1 albino mice (60 males and 60 females), were divided into 12 groups of equal sex; in part II, 90 CF-1 albino mice (45 males and 45 females), were divided into 9 groups of equal sex. Each group was labeled according to the concentration of methylcholanthrene which subsequently was to be applied to it. Each mouse approximately twelve weeks old, was placed in an individual metal cage constructed with a wire mesh front and bottom. Excreta pans were arranged to permit coprophagy to take place. The diet consisted of Purina Laboratory Chow and tap water, both given *ad libitum*. Food was placed in ample amounts on the floor of each cage and water was obtained through a glass drinking tube that was attached to a six-ounce bottle. The diet was supplemented once a week with lettuce and carrots for two successive weeks, followed by another two-week interval in which the supplement consisted of bread soaked in milk mixed with 2 ml. per quart of Vi-Penta drops.

Care was taken to eliminate all drafts and the temperature was maintained at 25° throughout the experiment. The cages were rotated before a window to standardize the amount of visible light to which the mice were exposed. This was done to conform with the observation of Morton, *et al.* (9), that light can influence tumor growth.

Two hundred and fifty milliliters of a 0.15% stock solution of methylcholanthrene in acetone were prepared. Aliquot portions of this stock solution were measured with syringe-adapted transfer pipets and added to the proper amount of acetone to make 30 ml. of solution ranging in concentration from 0.04 to 0.15% in increments of 0.01%. All measurements were made at room temperature.

In order to facilitate the handling of the methylcholanthrene solutions, ampuls were made and stored in a refrigerator at 0–5° until used.

* Received May 3, 1957, from Temple University, School of Pharmacy, Philadelphia, Pa.

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Presented to the Scientific Section, 4th Pan-American Congress of Pharmacy and Biochemistry, Washington, D. C., November, 1957.

For a quantitative study of the amount of carcinogen necessary to induce tumors a more exact method of application than the method of Cramer and Stowell (5) is required. In this experiment, micro-pipets, with a capacity of 0.2 ml., were employed. With these pipets, 0.02 ml. could be precisely measured and applied to the interscapular region.

EXPERIMENTAL

Heavy Neoprene gloves were used to protect the hands while handling the mice and apparatus. The mice were always grasped by the tail with long forceps and transferred to the left hand and held at the base of the tail by the thumb and index finger. In this position they were then placed under the micro-pipet for application of the carcinogen to the interscapular space.

Each mouse received 0.02 ml. of its designated concentration of methylcholanthrene solution twice a week applied to the interscapular region, which was first sheared with an electric clipper and then once each following week with sharp barber scissors.

Upon the appearance of the first growth, each animal was then carefully examined four times a week with a model 701-A Stanley magnifying glass. At the termination of both parts of the experiment, all tumors were drawn and recorded on special data sheets that gave all of the necessary information concerning each mouse that showed evidence of tumor inducement. Also, several mice with typical growths were chloroformed and the tumors excised and immersed in Bouin's fixative for histological examination.

At the termination of both phases of the experiment all pertinent data were assembled and placed in four tables. Only those tumors which measured at least one dimension (width *versus* height) of 1 mm. or greater were recorded. Growths which did not conform to this criterion were extremely difficult to identify as epitheliomas. Table I (Part I) and Table III (Part II) show the chronological induction time and the quantity in micrograms of methylcholanthrene applied to the mice through the exact day on which a tumor was recorded according to the above-mentioned specifications. Table II (Part I) and Table IV (Part II) contain the complete data with respect to the separate groups at the termination of each phase of the experiment and relate the total quantity of methylcholanthrene administered in mcg. with tumor incidence per group during the 10-week duration of each phase of the experiment.

TABLE I.—MICROGRAM QUANTITIES OF METHYLCHOLANTHRENE APPLIED UNTIL ONSET OF TUMORS

No. of Mice	Sex	Concn., % M.C.A.	No. of Applications	Total Admin., mcg.	Induction Time, Days
90	F	0.12	14	336	49
96	F	0.13	16	416	55
119	F	0.15	16	480	55
118	F	0.15	17	510	58
51	M	0.09	18	324	62
70	F	0.10	18	360	62
74	M	0.11	18	396	62
88	F	0.12	18	432	62
109	F	0.14	18	504	62
102	M	0.14	18	504	63
100	F	0.13	19	494	64
84	M	0.12	19	456	65
106	F	0.14	19	532	65
46	F	0.08	20	320	69
79	F	0.11	20	440	69
83	M	0.12	20	480	69
86	F	0.12	20	480	69
93	M	0.13	20	520	69
99	F	0.13	20	520	69
110	F	0.14	20	560	69
2	M	0.04	21	168	72
19	F	0.05	21	210	72
31	M	0.07	21	294	72
36	F	0.07	21	294	72
87	F	0.12	21	504	72
89	F	0.12	21	504	72
117	F	0.15	21	630	72

Epilation was noticed in most animals beginning about four to five days after the first application of all of the various solutions of methylcholanthrene. The epilation became more and more pronounced for about 18 days, at which time many of the animals showed evidence of practically complete epilation of the interscapular region and its vicinity to which the carcinogen had spread by capillarity. For the next few days, this nearly complete epilation persisted and was then followed by another period during which there occurred slight stimulation of hair growth for one week. The mice which exhibited marked epilation then underwent a resurgence of hair growth which left these animals with

TABLE II.—INCIDENCE OF TUMOR INDUCTION WITH BIWEEKLY APPLICATIONS OF VARIOUS CONCENTRATIONS OF METHYLCHOLANTHRENE TO MICE FOR 10.4 WEEKS

Concn. of Methylchol., %	Total Admin., mcg.	Effective Total, Mice/Group	No. Tumors/ Group	Incidence of Tumors, %/Group	Sex	
					M	F
0.04	168	10	1	10.0	1	
0.05	210	8	1	12.5		1
0.06	252	10	0	0.0		
0.07	294	10	2	20.0	1	1
0.08	336	8	1	12.5		1
0.09	378	7	1	14.3	1	
0.10	420	8	1	12.5		1
0.11	462	10	2	20.0	1	1
0.12	504	9	7	77.8	2	5
0.13	546	10	4	40.0	1	3
0.14	588	9	4	44.4	1	3
0.15	630	9	3	33.3		3

TABLE III.—MICROGRAM QUANTITIES OF METHYLCHOLANTHRENE APPLIED UNTIL ONSET OF TUMORS

No. of Mice	Sex	Concn., % M.C.A.	No. of Applications	Total Admin., mcg.	Induction Time, Days
81	M	0.12	15	360	53
117	F	0.15	15	450	53
88	F	0.12	15	360	54
43	M	0.08	17	272	58
110	F	0.14	17	476	60
105	M	0.14	18	504	63
90	F	0.12	19	456	64
65	M	0.10	19	380	67
79	F	0.11	19	418	67
86	F	0.12	19	456	67
101	M	0.14	19	532	67
114	M	0.15	19	570	67
113	M	0.15	20	600	69
36	F	0.07	21	294	72
40	F	0.07	21	294	72
61	M	0.10	21	420	72
78	F	0.11	21	462	72
85	M	0.12	21	504	72
87	F	0.12	21	504	72

Histopathological examination of several typical tumors revealed that these growths were true cancers of the type that are produced on the epidermis by methylcholanthrene, *viz.*, squamous cell carcinomas.

DISCUSSION

As shown in Tables II and IV, the results were similar in each phase (Part I and Part II) of the experiment. At the termination of each phase, the animals which received biweekly applications (on Tuesdays and Thursdays) of a 0.12% solution of methylcholanthrene in acetone gave evidence of at least 50% carcinogenesis. Each member of this group received a total of 504 mcg. of methylcholanthrene. Therefore, this total dose administered through 21 applications of 0.02 ml. of a 0.12% methylcholanthrene/acetone solution may be designated the MCD_{50} necessary to induce tumors on the epidermis of CF-1 mice. The tumors so induced measured at least one dimension (width *versus* height) of 1 mm. or greater.

The minimal carcinogenic $dose_{50}$ appears to be the optimal dose for tumor formation since both larger and smaller doses within the range of this experiment exerted a delay in the onset of carcinogenesis. It is extremely difficult to present an adequate explanation of this phenomenon since a paucity of fundamental knowledge regarding the intracellular action of methylcholanthrene exists in the literature. One might hypothesize in view of these results that a threshold exists for a maximal carcinogenic response. Doses which fail to attain this threshold perhaps are unable to cause an interaction with the intracellular receptors, a condition which may trigger off the transformation to malignancy. On the other hand, doses which surpass the threshold may inactivate the receptors by being present in greater amounts, thereby retarding the cancerization process.

The procedure outlined herein has been shown to yield results that are both constant and reproducible. This technique may be employed in future studies to determine various dose/response relationships for all of the other hydrocarbon carcinogens, with the possible clarification of some of the basic principles which concern the cancerization process.

There were 29 females and 17 males which developed measurable tumors during the course of the entire experiment. Therefore, it appears that

TABLE IV.—INCIDENCE OF TUMOR INDUCTION WITH BIWEEKLY APPLICATIONS OF VARIOUS CONCENTRATIONS OF METHYLCHOLANTHRENE TO MICE FOR 10.4 WEEKS

Concn. of Methylchol., %	Total Admin., mcg.	Effective Total, Mice/Group	No. Tumors/ Group	Incidence of Tumors %/Group	Sex	
					M	F
0.07	294	10	2	20.0		2
0.08	336	10	1	10.0	1	
0.09	378	10	0	0.0		
0.10	420	10	2	20.0	2	
0.11	462	10	2	20.0		2
0.12	504	10	6	60.0	2	4
0.13	546	9	0	0.0		
0.14	588	10	3	30.0	2	1
0.15	630	10	3	30.0	2	1

longer hair in the interscapular region than on any other area of their bodies.

Nine days after the first application of the respective solutions of methylcholanthrene, slight vasodilation of subcutaneous blood vessels was observed with the naked eye. This phenomenon persisted in most animals throughout the experiment, while some of the mice had small subcutaneous hemorrhages that often broke the surface of the epidermis.

Of the 210 animals employed in both phases of the experiment, only 19 died. Fourteen males and five females succumbed due to causes other than those of the experiment. The mice that died showed no signs of injured epidermis or hemorrhage in the interscapular region. The animals that died became progressively sicker and succumbed within a five to nine-day period. Only one mouse died after the appearance of a tumor.

Among the 42 control animals (21 males and 21 females), seven deaths were recorded, all of them males. At no time did a spontaneous tumor appear in any of the control animals. Also, by breeding several members from the control group, it was possible to check the appearance of spontaneous growths in the progeny. Through many successive generations there was no evidence of such spontaneity.

methylcholanthrene causes a higher tumor incidence in female mice under these conditions. Females may respond more readily due to the higher mitotic activity and thickness of the epidermis in the anterior dorsal region just prior to estrus (10).

The epilation and subcutaneous vasodilation caused by methylcholanthrene are in accord with the findings of Cramer and Stowell (5). The resurgence of hair growth in most animals is probably the stage in which hypertrophy and hyperplasia of the epidermal tissues occur. This, therefore, may be regarded as the period during which there is a transformation from the normal stage to one of malignancy, later developing into squamous cell carcinoma.

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Polyphosphoric Acid in the Bischler-Napieralski Reaction*

By JOSEPH G. CANNON† and GEORGE L. WEBSTER‡

The use of polyphosphoric acid in syntheses of 1-substituted 3,4-dihydroisoquinolines from N-acyl phenethylamines has been investigated, and a method of synthesis resulting in greatly improved yields has been developed. Two new heterocyclic bases have been prepared, and several previously unreported derivatives have been listed. A heterocyclic base not previously prepared from an N-acyl phenethylamine has been synthesized.

THE UTILITY of polyphosphoric acid as a dehydrating agent and as a cyclizing agent has been demonstrated repeatedly in the recent literature. The apparent versatility of this reagent suggested its use as the cyclizing agent for N-acyl phenethylamines in Bischler-Napieralski syntheses of 1-substituted 3,4-dihydroisoquinolines. Leonard and Boyer (1) prepared 1-methyl-3,4-dihydroisoquinoline in 20% yield from N-(β-phenethyl) cyanoacetamide, using a commercial polyphosphoric acid in the ratio of five grams to one gram of the amide. Snyder and Werber (2) cyclized acetyl phenethylamine by heating it with ten times its weight of a commercial polyphosphoric acid; they obtained a 23% yield of 1-methyl 3,4-dihydroisoquinoline. These workers similarly prepared 3,4-dihydroisoquinoline in 31% yield. Pratt, Rice, and Luckenbaugh (3) reported a 79% yield of crude 3,4-dihydroisoquinoline in a polyphosphoric acid condensation of N-formyl phenethylamine. Murakoshi (4) prepared 1-benzyl-3,4-dihydroisoquinoline in 20% yield by treating N-phenylacetyl phenethylamine with a mixture of phosphorus oxychloride and extemporaneously prepared polyphosphoric acid. Hey and Lobo (5) obtained a 39% yield of 1-benzyl-3,4-dihydroisoquinoline on refluxing N-phenylacetyl phenethylamine with polyphosphoric acid in xylene solution.

Literature reports concerning the general applicability of polyphosphoric acid to Bischler-Napieralski cyclizations of N-acyl phenethylamines are incomplete; therefore, a study was undertaken to evaluate the use of the reagent in the preparation of a series of 1-substituted 3,4-dihydroisoquinolines. In all reactions reported here, freshly prepared polyphosphoric acid was used in the ratio of 330 grams per 0.1 mole of amide; this was roughly equivalent to 10 to 15 grams of polyphosphoric acid per gram of the amide. Yields of 3,4-dihydroisoquinolines were, in every instance where comparison was possible, superior to those reported previously in the literature.

1-Isopropyl- and 1-isobutyl-3,4-dihydroisoquinoline have not been listed in the literature prior to this paper. In connection with other studies, a sample of 1-isobutyl isoquinoline was needed; this was also a new compound, and it was prepared by aromatization of the corresponding 3,4-dihydroisoquinoline. The identity of the aromatized compound was confirmed by comparison with a sample prepared by an entirely different method (6). The methiodides of

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all of the heterocyclic bases reported in this paper have not been previously listed in the literature.

EXPERIMENTAL

N-Acyl Phenethylamines.—Several methods were employed: (*a*) Condensation of the acid chloride with an equimolecular quantity of phenethylamine in the presence of an equivalent portion of pyridine; (*b*) the Schotten-Baumann Method; (*c*) refluxing the acid chloride with an equimolecular quantity of the amine in benzene solution; (*d*) refluxing equimolecular amounts of the acid and the amine. See Table I.

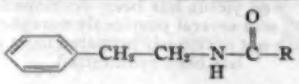
Polyphosphoric Acid.—One hundred and seventy-five grams of phosphorus pentoxide was weighed into a round-bottom flask, and to it was added in small amounts and with agitation 100 ml. of 85% orthophosphoric acid. The mixture was agitated for about one-half hour, until all solid matter had dissolved; it was then used immediately in the cyclization reactions.

3,4-Dihydroisoquinolines.—One-tenth mole of the N-acyl phenethylamine was added with thorough mixing to the freshly prepared polyphosphoric acid. The resulting homogeneous mixture, protected by a calcium chloride drying tube, was heated in an oil bath for three hours at a bath temperature of 200–205°.

While still hot, the viscous dark liquid was poured over an excess of ice contained in a large round-bottom flask, and the reaction flask was rinsed with small portions of water, which were added to the ice-water mixture. A concentrated solution of sodium hydroxide was then added with agitation and cooling until the contents of the flask were strongly alkaline to litmus. This mixture was then steam distilled, and the distillate was extracted three times with benzene. The benzene extracts were combined and dried over anhydrous magnesium sulfate, filtered, and the benzene was removed on a steam bath by use of a water aspirator. The dark residue was then distilled under reduced pressure. 1-Phenyl- and 1-benzyl-3,4-dihydroisoquinoline were not steam distilled; rather, the basified reaction mixtures were extracted several times with benzene to separate the products. See Table II.

1-Isobutyl Isoquinoline.—Ten grams of 1-iso-

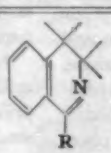
TABLE I.—ACYL PHENETHYLAMINES



"R"	b. p. or m. p.[a]	mm.	Yield, %	Method of Prepn.	Formula	Analysis[d]
H	146–152	0.8	67	[d]	....	....
CH_3	128–135	1	55	[a]	....	....
C_2H_5	150–155	0.8	65	[a]	....	....
n-C_3H_7	155–160	0.8	78	[a]	....	....
iso-C_3H_7	80–81[a]	..	45	[a]	$C_{12}H_{17}NO$	N Calcd., 7.34; Found, 7.15
n-C_4H_9	147–152	0.15	62	[a]	....	....
iso-C_4H_9	57–59[a]	..	71.5	[a]	$C_{12}H_{19}NO$	N Calcd., 6.82; Found, 6.79
n-C_5H_{11}	154–156	0.03	40	[a]	$C_{14}H_{21}NO$	N Calcd., 6.40; Found, 6.58
	32–34[a]					
C_6H_5	115–116[b]	..	90	[b]	....	....
Benzyl	87–89[c]	..	75	[c]	....	....
Cyclohexyl	97–98[c]	..	39	[c]	....	....

[a] Recrystallized from aqueous ethanol.
[b] Recrystallized from 95% ethanol.
[c] Recrystallized from benzene.
[d] Compounds for which an analysis is given are those not previously reported in the literature. Analyses for nitrogen by Drs. Weiler and Strauss, Oxford, England.
[e] All melting points uncorrected.

TABLE II.—3,4-DIHYDROISOQUINOLINES



"R"	b. p. or m. p.[a]	mm.	Yield, %	n_D^t	Formula	Analysis[b]
H	75–77	1	75	1.5815 (24°)	....	.....
...Picrate	173–175	..	..	..	.....	.....
...Methiodide	125–126	..	..	..	$C_{10}H_{12}NI$	N Calcd. 5.13; Found, 4.90

TABLE II.—(*continued*)

CH_3	70–76	1	82	1.5738 (20°)	....	.....	
...Picrate	188–192	..	..	..	....	.....	
...Methiodide	200–202	..	..	..	$C_{11}H_{14}NI$	N Calcd., 4.88; Found, 4.70	
C_2H_5	79–86	1	77.2	1.5632 (25°)	....	.....	
...Picrate	195–197	..	..	..	....	.....	
...Methiodide	113–115	..	..	..	$C_{12}H_{16}NI$	N Calcd., 4.64; Found, 4.35	
n-C_3H_7	112–113	1	71.8	1.5532 (22°)	....	.....	
...Picrate	162–165[a]	..	..	..	....	.....	
...Methiodide	132–134	..	..	..	$C_{13}H_{18}NI$	N Calcd., 4.44; Found, 4.29	
iso-C_3H_7	85–95	1.5	62.3	1.5453 (22°)	....	.....	
...Picrate	171–172	..	..	..	$C_{18}H_{18}N_4O_7$	N Calcd., 13.95; Found, 13.96	
...Methiodide	202–205	..	..	..	$C_{13}H_{18}NI$	N Calcd., 4.44; Found, 4.33	
n-C_4H_9	87–93	0.15	82.8	1.5443 (25°)	....	.....	
...Picrate	149–150	..	..	..	....	.....	
...Methiodide	112–113	..	..	..	$C_{14}H_{20}NI$	N Calcd., 4.26; Found, 4.32	
iso-C_4H_9	69–72	1	68	1.5448 (20°)	$C_{13}H_{17}N$	N, Calcd., 7.48 Found, 7.34	
...HCl	126–129	..	..	..	$C_{13}H_{18}NCl$	Cl Calcd., 15.84; Found, 15.92	
...Picrate	150–153	..	..	..	$C_{19}H_{20}N_4O_7$	N Calcd., 13.52; Found, 13.40	
...Methiodide	108–110	..	..	..	$C_{14}H_{20}NI$	N Calcd., 4.26; Found, 4.08	
n-C_5H_{11}[d]	102–106	0.15	67.5	1.5383 (25°)	....	.....	
...Picrate	111–112	..	..	..	....	.....	
...Methiodide	70–71	..	..	..	$C_{15}H_{22}NI$	N Calcd., 4.08; Found, 4.18	
Cyclohexyl	114–120	0.15	70	1.5568 (24°)	....	.....	
...Picrate	169–170	..	..	..	....	.....	
...Methiodide	181–183	..	..	..	$C_{16}H_{22}NI$	N Calcd., 3.95; Found, 4.08	
C_6H_5	128–138	1	79	1.6295 (25°)	....	.....	
...Picrate	173–175	..	..	..	....	.....	
...Methiodide	188–190	..	..	..	$C_{16}H_{16}NI$	N Calcd., 4.01; Found, 3.81	
Benzyl	132–140	0.01	84	1.6228 (25°)	....	.....	
...Picrate	180–182	..	..	..	....	.....	
...Methiodide	205–207	..	..	..	$C_{17}H_{18}NI$	N Calcd., 3.87; Found, 3.83	

[a] All melting points uncorrected.
[b] Compounds for which an analysis is given are those not previously reported in the literature. Analyses for nitrogen by Clark Microanalytical Laboratory, Urbana, Ill., and Drs. Weiler and Strauss, Oxford, England.
[c] Späth, Berger, and Kuntara, *Ber.*, **63**, 134(1930) reported m. p. 173–174.
[d] Prepared by Broderick and Short, *J. Chem. Soc.*, **1951**, 1343 from N-2 phenethyl benzamidinium chloride. Synthesis from the acyl phenethylamine not previously reported.

butyl-3,4-dihydroisoquinoline was heated with an approximately equal weight of freshly prepared Raney nickel until the mixture began to boil. It was then cooled, filtered, and the filter paper and its contents were thoroughly washed with ether, which was added to the filtrate. The ether was removed from the filtrate on a steam bath and the dark residue was distilled under reduced pressure, b. p. 129–131° (7.5 mm.). Yield, 3.24 Gm. (32.8%) of a light yellow liquid that soon darkened, n_{D}^{25} 1.5539. Picrate, recrystallized from absolute ethanol, m. p. (uncorrected) 168–171.°

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Vitamin B_{12} in the Presence of Vitamin B_1 and Niacinamide in Aqueous Combinations*

By ANA S. GAMBIER and ERWIN P. G. RAHN

New facts about the physical and chemical stability of B-complex aqueous solutions containing vitamin B_{12} are reported. Conditions concerning the preparation of such combinations are conclusively established. Limitations of artificial aging tests are given.

IN A RECENT PAPER (1) several statements were made on the stability of vitamin B_{12} in the presence of other B-complex components in aqueous solutions and, in addition to the problem of chemical stability, some physical changes were pointed out. Through further investigations it was possible to confirm more definitely the indispensable conditions to stabilize, chemically and physically, aqueous combinations of vitamins B_1, B_{12} and niacinamide.

COMMENTS

Concentration.—Gunsberg, *et al.* (2, 3), were the first to point out the importance of the relative concentrations of vitamin B_{12} and other components. They worked with solutions which had a higher proportion between the vitamin B_1 and vitamin B_{12} concentrations than 500 to 1, while Macek and Feller (4, 5) made their studies using such proportions.

Searches conducted to establish the limits of these proportions, previously considered by us to be ideal at 60 to 1 (1), led us to work with solutions containing vitamin B_1 and vitamin B_{12} in proportions as high as 10,000 to 1 (Table I, solutions 1 to 8). We found that in proportions of 120 and 500 to 1 the losses were negligible at room temperature after a short period of time and after a three-week exposure to 37° they were 12.2–15.4% and 23.8–26.7% respectively (Table I, solutions 3 and 5). For the proportions of 5,000 to 1 and 10,000 to 1 we found losses of 70.1% and more than 95% after a three-week exposure to 37° (Table I, solutions 7 and 8). Thus, the higher the proportions the greater the inactivation of vitamin B_{12}.

These results agree with those of Macek and Feller (4, 5) and Gunsberg, *et al.* (2, 3), and allowed us to conclude that considerably higher proportions than 120 to 1 between vitamin B_1 and vitamin B_{12} are not recommended for aqueous combinations of vitamin B_{12} with vitamin B_1 in the presence of other B-complex components.

Purity of Components.—All raw materials must be of the highest purity. Vitamin B_1, particularly, must be carefully tested "for parenteral use" as described in the experimental part. By using two pure U. S. P. B_1 vitamins, purchased on the market from different sources and both being "for parenteral use" but one of them not specifically retested just before processing, results as demonstrated by solutions number 4 and 6 compared to number 5 and 7 (Table I) may occur.

pH Value.—Our previous experiences (1) and the observations of Macek and Feller (4, 5) seem to indicate that the ideal range of pH is from 3.0 to 4.0. We obtained the best results at pH 3.3.

Product-Processing.—We sterilized the solutions by filtration through Chamberland L5 micro-porous porcelain candles. In addition to all the care previously described (1), the individual filtration of each vitamin is very important and is essential for vitamin B_1 and niacinamide.

"Liquid Volume to Air Volume" Ratio.—It was defined by us that fading of B-complex solutions containing vitamin B_{12} is a neat characteristic of vitamin B_{12} inactivation, but its darkening, as a rule, does not prove the same thing (1). Only negligible differences were observed on physicochemical (6) and microbiological (7) vitamin B_{12} assay results in a lot of two samples, one of which was darkened deeply and the other with slight physical changes. Both samples were prepared from one solution and tested under the same conditions (Table I, solutions 1 and 3). The darkening, however, was the greatest trouble we had.

We observed a definite influence of the amount of air present over the solutions in the ampuls on the physical stability regarding discoloration. Different ratios were used between the liquid volume filled in the ampuls and the air volume (or air space) present in the sealed ampuls. We found that the darkening of high concentrated B-complex aqueous combinations containing vitamin B_{12} increases in the presence of decreasing amounts of oxygen (air). Actually, stable aqueous combinations of vitamin B_{12} and other B-complex components, from a physical as well as chemical point of view, were possible only when a "liquid volume to air volume" ratio of 1.73 to 1.00 in the ampuls was observed (Table I, solutions 1 to 5).

In the course of our work, in order to emphasize the above mentioned points, we had the opportunity of proving again, by parallel microbiological assays, the applicability of Marsh and Kuzel's vitamin B_{12} assay method (6) in stability studies of aqueous combinations of vitamin B_{12} and other ionizable components (Table I, solutions 1 and 3). Limitations

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The microbiological assays given in this paper were made in the laboratory of the Biochemical Department, College of Pharmacy, University of São Paulo, Brazil, by Miss Aurora Leal, Assistant to Professor Henrique Tastaldi, to whom the authors are gratefully indebted.

are given by the concentration of vitamin B_{12} and the other components. This is the reason why we used the microbiological assay method (7) in the more diluted and disproportionated solutions (Table I, solutions 5 to 8).

On the other hand, the artificial aging test conditions must be carefully chosen. When aqueous combinations of vitamin B_1, B_6, and B_{12} are maintained at 45° for three weeks, they suffer similar losses such as those aged for 12 m.r.t.[1] For the artificial aging tests of vitamin B_1, vitamin B_{12} and niacinamide aqueous combinations, we consider that the best temperature range is between 37° and 40°. We used 37° and suppose that a three-week exposure gives results comparable with those to be expected after 12 m.r.t. (Table I, solution 3 after three weeks 37° and after 3, 6, and 9 m.r.t.). The presence of niacinamide in these solutions accelerates the vitamin B_1 decomposition and the resulting product (the thiazole moiety (1,5)) promotes the decomposition of vitamin B_{12}. This vitamin B_{12}

[1] m.r.t. = months at room temperature.

TABLE I.—B-COMPLEX SOLUTIONS CONTAINING VITAMIN B_{12} AT pH 3.3

Soln. No.	Vit. B_1 to Vit. B_{12} proportion	Components → Quantities per cc. →	Vit. B_1, mg.	Vit. B_6, mg.	Niacinamide, mg.	Vit. B_{12}, mcg.	Loss, %	Liquid to Air Volume Ratio	Physical Changes
1	120:1	Theoretical content	60	27.5	..	525	..	.....	..
		Initial assay	..	27.5	..	511[b]	..	.....	..
		After 6 m.r.t.[a] and then submitted to 3 weeks 37°	..	..	..	433[b]	15.3	1.73:1.00	[d]
						433[c]	15.3	1.73:1.00	[d]
						457[b]	10.6	2.76:1.00	[g]
						462[c]	9.6	2.76:1.00	[g]
2	120:1	Theoretical content	60	27.5	..	525	..	.....	..
		Initial assay	60.5	27.7	..	511[b]	..	.....	..
		After 2 m.r.t. and then submitted to 3 weeks 45°	58	26.2	..	489[b]	4.4	1.73:1.00 and 2.76:1.00	[d]
		After 2 m.r.t. and then submitted to 6 weeks 45°	56.2	28.2	..	385[b]	24.7	1.73:1.00 and 2.76:1.00	[g]
		After 6 m.r.t.	..	..	..	484[b]	5.3	1.73:1.00 and 2.76:1.00	[d]
		After 6 m.r.t. and then submitted to 3 weeks 37°	55	28.3	..	469[b]	8.3	1.73:1.00	[d]
			55	26.9	..	479[b]	6.3	2.76:1.00	[g]
3	120:1	Theoretical content	60[e]	27.5	55	500	..	.....	..
		Initial assay	53.1	28.0	54	470[b]	..	.....	..
						490[c]	..	.....	..
		After 3 weeks 37°	..	..	..	413[b]	12.2	1.73:1.00 and 2.76:1.00	[d]
						415[c]	15.4	1.73:1.00 and 2.76:1.00	[d]
		After 3 weeks 45°	..	..	..	381[b]	19.0	1.73:1.00	[d]
						372[c]	24.1	1.73:1.00	[d]
						367[c]	25.2	2.76:1.00	[g]
		After 3 m.r.t.	..	..	..	487[c]	0.7	1.73:1.00 and 2.76:1.00	[d]
		After 6 m.r.t.	..	..	..	461[b]	1.9	1.73:1.00	[d]
						...	..	2.76:1.00	[g]
		After 9 m.r.t.	..	..	..	417[c]	15.9	1.73:1.00	[d]
						...	..	2.76:1.00	[g]
4	500:1	Theoretical content	60[f]	27.5	55	120	..	.....	..
		Initial assay	..	..	..	113[b]	..	.....	..
		After 3 m.r.t.	..	..	..	73[c]	35.4	1.73:1.00 and 2.76:1.00	[h]
		After 3 weeks 37°	..	..	..	<5[c]	>95	1.73:1.00 and 2.76:1.00	[i]
5	500:1	Theoretical content	60[e]	27.5	55	120	..	.....	..
		Initial assay	59.4	28.7	56	126[b]	..	.....	..
						122[c]	..	.....	..
		After 3 weeks 37°	..	..	..	91.5[c]	23.8	1.73:1.00	[d]
						88[c]	26.7	2.76:1.00	[g]
		After 3 weeks 45°	..	..	..	66.5[c]	44.6	1.73:1.00 and 2.76:1.00	[g]
		After 3 m.r.t.	..	..	..	120[c]	0	1.73:1.00 and 2.76:1.00	[d]
6	5,000:1	Theoretical content	60[f]	27.5	55	12	..	.....	..
		Initial assay	..	..	..	12.6[c]	..	.....	..
		After 2 m.r.t.	..	..	..	...	..	1.73:1.00 and 2.76:1.00	[j]
		After 3 weeks 37°	..	..	..	<1[c]	>90	1.73:1.00 and 2.76:1.00	[i]
7	5,000:1	Theoretical content	60[e]	27.5	55	12	..	.....	..
		Initial assay	59.4	28.7	57.5	12.2[c]	..	.....	..
		After 3 weeks 37°	..	..	..	3.58[c]	70.1	1.73:1.00 and 2.76:1.00	[j]
8	10,000:1	Theoretical content	60[e]	27.5	55	6	..	.....	..
		Initial assay	60.4	28.7	52.5	5.5[c]	..	.....	..
						6.2[c]	..	.....	..
		After 3 weeks 37°	..	..	..	<0.25[c]	>95	1.73:1.00 and 2.76:1.00	[i]

Vit. B_1—Thiamine hydrochloride
Vit. B_6—Pyridoxine hydrochloride
Vit. B_{12}—Crystalline vitamin B_{12} U. S. P.
[a] m.r.t. = Months at room temperature.
[b] Physico-chemical assay (6).
[c] Microbiological assay (7).
[d] Without any noticeable physical change.
[e] Vit. B_1 "for parenteral use" type *A*.
[f] Vit. B_1 "for parenteral use" type *B*.
[g] Accentuated uniform darkening
[h] Slight color loss.
[i] Complete loss of color.
[j] Accentuated uniform color loss.

decomposition becomes greater with the higher the temperature and longer the time of exposure.

EXPERIMENTAL

All recommendations for raw materials and apparatus to be used should be exactly the same as previously described (1). The assays of the raw materials were performed according to the U. S. P. standards. An additional qualitative test for vitamin B_1 must be accomplished: only raw materials giving a completely clear and colorless 30% solution in double distilled water observed in a flint glass cylinder of at least 15 cm. diam. must be used. For the individual filtration of the different components in an all glass or glass-lined apparatus, a special assembly for vacuum filtration with a lot of micro-porous porcelain candles was designed.

Each candle, free from heavy metal traces (8), was previously washed with 1,000 cc. of 0.1 *N* hydrochloric acid, subsequently with 1,000 cc. of hot double-distilled water, and finally with cold distilled water until all chloride ions were removed. After heat sterilization of the apparatus, the filtration of the different vitamin solutions was made by suction. One candle must be used for vitamin B_1 and another for niacinamide. Vitamin B_6 and vitamin B_{12} can be filtered through the same candle. The pH of the different vitamin solutions was previously ascertained by partial neutralization with an experimentally determined quantity of analytical reagent grade sodium bicarbonate so that the final solutions to be subdivided in the ampuls has the expected value. First we prepared and filtered a 30% solution of the vitamin B_1, adjusted to pH 3.3. We then filtered through another candle a 20% solution of the niacinamide, adjusted to pH 3.3 with analytical reagent grade hydrochloric acid. Finally we filtered a 10% solution of the vitamin B_6 containing the vitamin B_{12}, adjusted to pH 3.3. After every filtration, each candle was washed with double-distilled water until desired volume.

The filling of the ampuls must be made so that the ratio between the liquid volume and the air volume present in the sealed ampuls is 1.73 to 1.00.

For the microbiological assay of vitamin B_{12}, the U. S. P. method, using *Lactobacillus leichmanii*, was applied (7). The other analyses were made by the same methods as previously indicated (1).

The final sterility of all solutions was bacteriologically confirmed.

RESULTS AND DISCUSSION

It was possible to obtain chemically stable aqueous combinations containing vitamins B_1, B_{12}, and niacinamide if a certain pH value and a maximum proportion of 120 to 1 between vitamin B_1 and vitamin B_{12} are observed. Rapid inactivation of vitamin B_{12} occurs in solutions which have a proportion of 5,000 to 1 or 10,000 to 1, even when refined pharmacotechnical methods are applied for the preparation of these parenteral forms.

The darkening is considerably increased at higher pHs [4.0 and 4.5, solutions number 9 and 12 described in Table II of the previous paper (1)] and at high temperature levels (37° and 45°, Table I, solutions 3 and 5). Different air volumes present over the solutions in the ampuls also influence definitely the physical stability of these combinations. Using liquid volume to air volume ratios from 2.76 to 1.00 until 1.73 to 1.00 we obtained different degrees of darkening, from deep discolorations to no color changes. Characteristic results are demonstrated by Table I, solutions 1, 2, 3, and 5. Lower ratios than 1.73 to 1.00, i. e., presence of greater excesses of air in the sealed ampuls may cause a white precipitate. At a liquid volume to air volume ratio of 1.73 to 1.00 neither darkening nor precipitation were observed (Table I, solution 2 after 6 m.r.t. and then three weeks 37° exposure, solution 3 after three weeks 45° exposure and after 9 m.r.t., and solution 5 after three weeks 37° exposure).

The results regarding discoloration of B-complex aqueous combinations containing vitamin B_{12} are contrary to the well-known observations of darkening of B-complex aqueous combinations without vitamin B_{12} as a result from thiamine decomposition influenced by the air present over the solutions. Even at a pH 3.3, vitamin B_1, vitamin B_6, and niacinamide aqueous combinations darken in the presence of large air volume in the ampuls, while in the presence of small quantities of air they are perfectly stable (9). Nevertheless, these combinations in the presence of vitamin B_{12} and in the concentrations we worked, gave us the results previously discussed.

The chemical problems related to these physical changes are still being studied.

Summarizing, the important factors to obtain stable aqueous combinations on these vitamins are: (*a*) the relative vitamin B_1 concentration present in combinations of vitamin B_{12} with other B-complex components, (*b*) the pH value, (*c*) the "liquid volume to air volume" ratio in the ampuls or vials.

The general interest to perform artificial aging tests at temperatures of 100° and 120° in this type of solution seems to disappear in view of the achieved high vitamin B_{12} losses. Tests performed for a few weeks at only 37°, 40°, and 45° seem to give more conclusive stability results.

The individual filtration of the different vitamins is necessary to improve the chemical and physical stability of these solutions. The decomposition of vitamin B_1 in the presence of niacinamide is increased by the candles, in spite of the previous washing treatment where at least all calcium ions were removed.

Vitamin B_6 does not interfere in the stability of these combinations if the indicated product processing rules are strictly observed.

SUMMARY

1. The importance of the relative proportion between vitamin B_1 and vitamin B_{12} was pointed out in view of the stability of aqueous combinations containing vitamin B_1, vitamin B_{12}, and niacinamide.

2. The darkening of B-complex solutions containing vitamin B_{12} in high concentrations was avoided by increasing the air content over the solutions in the ampuls.

3. Special emphasis was given on the importance of the processing rules concerning the preparation of the discussed parenteral solutions.

4. Good stability of vitamin B_{12} was obtained even in presence of 60 mg./cc. of vitamin B_1 and 55 mg./cc. of niacinamide.

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A Chromatographic-Spectrophotometric Method for the Separation and Determination of Colchicine*

By STANISLAUS J. SMOLENSKI, FRANK A. CRANE, and RALPH F. VOIGT

An improved procedure for the extraction, separation, and purification of colchicine from plant tissue is presented. The method makes use of a simple separation and purification of colchicine by adsorption on two different chromatographic columns, eluting these with different solvents and determining the purified alkaloid colorimetrically.

A NUMBER OF METHODS have been devised for the extraction, separation, purification, and assay of colchicine. The extraction of the alkaloid has been carried out by making use of water (1–3), alcohol (4–8), wax and paraffin-wax (for the removal of resin) (4, 5), and a Soxhlet apparatus (8, 9).

Separation of colchicine from corm and seed tissue has been carried out by using chloroform (1–10). Determinations were made (a) gravimetrically, after extracting with methyl alcohol, shaking out the colchicine from chloroform in a separatory funnel (1, 6, 7); (b) polarigraphically, after extraction with water (3); and (c) colorimetrically (10, 11). Purification has been carried out on a chromatographic column, (12) making use of chloroform as the eluent.

In this study the alkaloid was extracted by a one per cent solution of ethyl alcohol in ethyl acetate after the removal of fixed oils by petroleum ether in a Soxhlet apparatus. This method makes use of a simple separation and purification of colchicine by adsorption on two successive chromatographic columns, eluting these with different solvents and determining the purified alkaloid colorimetrically. For this purpose Struve's color reaction (13) was adapted and the developed color was stable for at least three hours.

According to Struve (13), on addition of concentrated nitric acid colchicine changes from a yellow powder to a violet solution, which further changes, in a few minutes to brown-red; and then upon the addition of water, to yellow. On the further addition of sodium hydroxide T. S. the color becomes orange-red. This color test was applied in this laboratory to 28 other alkaloids or their salts, and none of them gave the identical reaction to colchicine in all three phases, i. e., with nitric acid, water, and sodium hydroxide T. S. For this reason Struve's color reaction was used throughout this work during the separation and purification of colchicine for testing the completion of extraction, and was further adapted for the quantitative spectrophotometric assay.

EXPERIMENTAL

Reagents Used.—1. Solvents used in extraction were petroleum benzin, anhydrous ethyl acetate containing 1% ethyl alcohol, and benzene C. P. 2. Chromatograms were prepared in glass chromatographic tubes using (a) alumina-oxide-Alcoa, mesh 80–200, grade F-20 (not reacted before use), protected at both ends by a thin layer of Hyflo-Supercel (Johns-Manville), and (b) silicic acid gel (ppt. $SiO_2 \cdot H_2O$) analytical reagent, Mallinckrodt, immersed in chloroform. 3. For eluting alumina column chromatograms, three successive solutions were used: (a) Chloroform U. S. P. XIV, (b) 4% solution of methyl alcohol U. S. P. XIV in Chloroform U. S. P. XIV, (c) 10% solution of methyl alcohol U. S. P. XIV in Chloroform U. S. P. XIV.

For eluting silica gel chromatograms, five successive solvents were employed: (a) chloroform, U. S. P. XIV, (b) 1% solution of methyl alcohol in chloroform U. S. P. XIV, (c) 2% solution of methyl

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alcohol in chloroform, (d) 10% solution of methyl alcohol in chloroform, and (e) 20% solution of methyl alcohol in chloroform. 4. Solutions used for the color reaction determining the presence of colchicine included concentrated nitric acid (reagent grade), distilled water, and sodium hydroxide T. S. This test was applied wherever colchicine was to be detected.

Extraction.—A representative sample of 10.0 Gm. of colchicum seed, reduced to a coarse powder (20-mesh) is extracted for four hours with 100 ml. of petroleum benzin in a Soxhlet apparatus. This solvent containing fats and resins is removed and the flask is replaced by one containing 100 ml. of anhydrous ethyl acetate with 1 ml. of ethyl alcohol. The powder is macerated for eight to twelve hours followed by continuous extraction for eight hours.

At the end of this period, 2 ml. of the solvent are removed from the Soxhlet extractor, evaporated to dryness, and tested for completeness of extraction of colchicine. The crude extract containing colchicine is concentrated to dryness by distillation. Five ml. of methyl alcohol are added to the residue and evaporated to dryness to remove all traces of ethyl acetate. The dried residue is taken up in 3 ml. of methyl alcohol, which is poured into a separatory funnel attached to a chromatographic column. The alcohol insoluble fraction remaining in the Soxhlet flask is washed by several portions of benzene to total 100 ml., which is finally collected in the separatory funnel.

Adsorption Columns.—For the first chromatographic column an adsorption tube 220 mm. in length and 20 mm. in inner diameter with a ground-glass joint and sealed-in perforated disk is employed. A 13-mm. layer of Hyflo-Supercel is placed in the bottom of the tube, upon which 20.0 Gm. of the alumina adsorbent are packed. Above the evenly distributed 90-mm. column (±5 mm.) a thin layer (0.25 Gm.) of Hyflo-Supercel is added to prevent the column from cracking; and then the column is washed with 100 ml. of benzene.

A second chromatographic column makes use of the same type of tube. Two filter paper disks are placed on the top of the perforated glass, upon which 15.0 Gm. of silicic acid mixed with 50 ml. of chloroform are poured along a glass rod, while holding the filter paper in place. The column is left to settle for a few minutes and then stirred thoroughly in order to remove air bubbles and particles that are not gel-like, and also insure an even surface at the top of the column. After ten to fifteen minutes slight suction is applied and the gel-like adsorbent settles, making a column 110 mm. (±5 mm.) x 20 mm. Suction is controlled so as to avoid drying of the silicic acid. This can be detected as a white powder in the gel-like column.

A carefully prepared column retains the desired consistency throughout the entire process of elution. Dry particles decrease the rate of flow; and if the entire column dries out, elution becomes impossible.

The combined alcohol-benzene solution of colchicine present in the separatory funnel is passed through the first column. A chromatogram is developed which consists of three poorly separated bands. The top brown band is about 3 mm. wide, the middle reddish-brown band is about 4 mm. wide, and the lowest band which contains colchicine is about 3 mm. wide and yellow in color.

The elution of colchicine is carried out in three stages with: (a) 100 ml. of chloroform; (b) 200 ml. of a 4% solution of methyl alcohol in chloroform; and (c) 200 ml. of a 10% solution of methyl alcohol in chloroform. After complete elution of the yellow band, 20-drop samples of the filtrate contain no colchicine.

The filtrate obtained from the separation is concentrated by distillation to about 1 ml. and transformed quantitatively in chloroform to a 100-ml. beaker, then evaporated to dryness on a water bath. The process of evaporation is repeated twice with 5-ml. portions of chloroform each time to remove all alcohol and benzene.

The washed and dried residue obtained from the filtrate of the first separation is dissolved in 10 ml. of chloroform and poured into the separatory funnel mounted above the second column. The beaker which contained this residue is washed with small portions of chloroform totaling 50 ml. and the combined solutions are passed through the silicic acid column. A chromatogram is developed consisting of the following bands (from top to bottom): 1. yellow, 2. red, 3. colorless, 4. red, 5. colorless, and 6. yellow-green-yellow.

Bands 2, 4, and 6, which contain impurities, are eluted with 100 ml. of chloroform, then with solutions of methyl alcohol in chloroform—first 125 ml. of 1% and finally 50 ml. of 2%. The colchicine present in band 1 remains at the top of the column and moves only slightly. During this process of elution, eluents consisting of more than 2% of methyl alcohol in chloroform should be avoided because higher concentrations of alcohol disturb the order of bands.

Colchicine is eluted as the final component, making use of 150 ml. of a 10% solution of methyl alcohol in chloroform. It is possible to increase the rate of elution with concentrations as great as 20%. Again, samples are taken to test completion of elution.

The eluate containing the colchicine is evaporated to dryness on a water bath. Ten ml. of methyl alcohol are added and evaporated in two successive steps to remove the chloroform.

Development of the Standard Curves.—The adsorption curves (Fig. 1) and the colchicine standard curve (Fig. 2) were derived by the following procedure: Samples of 0.5 mg., 1.0 mg., 2.5 mg., 3.0 mg., and 4.5 mg. of colchicine U. S. P. XIV were placed in 10-ml. volumetric flasks and dissolved in 0.1 ml. of concentrated nitric acid. A dark purple-blue-purple (PB-P) color (14) developed which changed to red-purple-red (RP-R), then finally red-yellow (RY), by the end of thirty minutes. This acid solution was washed with water containing 2.5 ml. of sodium hydroxide T. S., thereby developing a red color.

The flask was filled to volume with distilled water. The absorbances of these solutions were measured on the Beckman DU spectrophotometer, and these were used in plotting the absorption curves (Fig. 1). Single peaks of absorbance occurred at 350 millimicrons. The color densities for each concentration were then plotted and used to prepare the standard curve (Fig. 2).

Determination of Colchicine from Plant Tissue.—The residue from the chromatographic separations is transferred to a 100-ml. volumetric flask by means of methyl alcohol and made up to volume. Ten-ml. samples of this solution are measured into weighing bottles, evaporated carefully to dryness on a water bath, and dried in an oven for sixteen hours at 105°. One-tenth ml. of concentrated nitric acid is added to the residue and allowed to stand for thirty minutes in order to develop the color. This acid solution is washed into a 50-ml. volumetric flask containing 2.5 ml. of sodium hydroxide T. S. The flask is filled to volume with distilled water, mixed thoroughly, and allowed to stand for fifteen minutes.

In order to have a solution within the proper concentration range, 3-ml., 5-ml., and 8-ml. aliquots are transferred to 10-ml. volumetric flasks and filled to the mark with water. The optical density of these solutions is read on the Beckman DU spectrophotometer at 350 mμ and the amount of colchicine is determined from the standard curve.

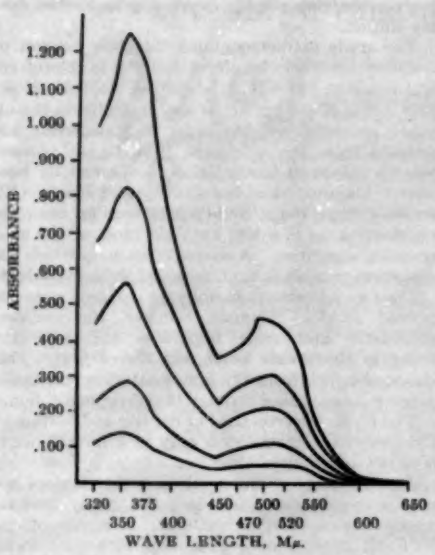

Fig. 1.—Absorption curves for colchicine following reaction with HNO_3, H_2O, and NaOH T.S. Amounts of colchicine present (reading from bottom to top of graph): I. 0.05 mg. in 10 ml. II. 0.1 mg. in 10 ml. III. 0.2 mg. in 10 ml. IV. 0.3 mg. in 10 ml. V. 0.45 mg. in 10 ml.

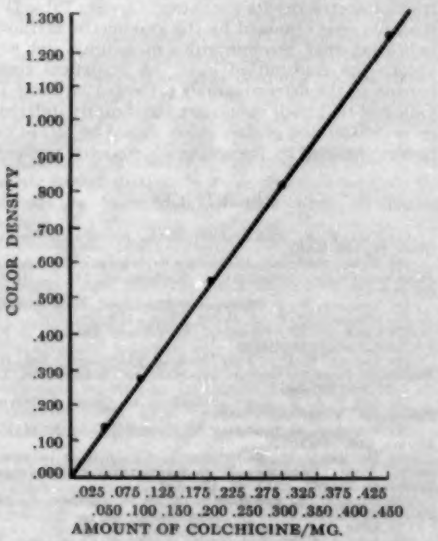

Fig. 2.—Standard curve obtained by plotting the spectrophotometric color density at 350 mμ against amount of colchicine in mg.

RESULTS

The sample of extracted colchicum seed mentioned above was divided into aliquots which were determined by both spectrophotometric and gravimetric methods. The results of these determinations are shown in Table I. The method was further compared with the official N. F. IX method and results are shown in Table II.

TABLE I.—RESULTS OF THE SPECTROPHOTOMETRIC AND GRAVIMETRIC METHODS FOR DETERMINING COLCHICINE IN COLCHICUM SEEDS

Sample number	Spectrophotometric method, mg./Gm. of seed	Gravimetric method, mg./Gm. of seed
1	3.0	3.3
2	3.0	3.6
3	3.1	3.4
4	3.0	4.3
5	3.1	3.7
6	3.0	3.8
7	3.6	3.7
8	3.6	3.6
9	3.2	3.2
10	3.1	3.3
11	3.0	3.3
12	3.0	3.1
Mean	3.1	3.5

TABLE II.—YIELD OF COLCHICINE OBTAINED BY THE N. F. IX METHOD AND BY THE EXPERIMENTAL METHODS (SPECTROPHOTOMETRIC AND GRAVIMETRIC)

Methods	Number of Determinations	Colchicine from 10 Gm. Colchicum Seed in mg.	Standard Deviation
N. F. IX	2	29.3	1.30
Experimental			
Spectrophotometric	12	31.6	2.40
Gravimetric	12	35.2	3.35

Sensitivity of method.—In order to test the degree of sensitivity of the method, a number of samples of colchicine were weighed, dissolved in the solvents mentioned above, and determined spectrophotometrically. The results are listed in Table III.

Determinations of Colchicine (U. S. P. XIV) were made by spectrophotometric and gravimetric methods, results of which are listed in Table IV.

TABLE III.—SPECTROPHOTOMETRIC DETERMINATION OF UNKNOWN QUANTITIES OF COLCHICINE[a]

Sample Number	Colchicine Taken, mg.	Colchicine Determined, mg.	Per Cent
1	5.2	5.0	96.1
2	2.1	2.0	95.7
3	2.5	2.4	96.0
4	2.5	2.5	100.0
5	1.0	1.1	110.0
6	1.0	1.0	100.0
7	1.4	1.4	100.0
8	0.2	0.2	100.0
9	4.5	4.4	97.8
10	1.1	1.2	109.0

[a] Obtained from Mallinckrodt Chemical Works.

TABLE IV.—DETERMINATION OF COLCHICINE, U. S. P. XIV[a]

Colchicine Taken, mg.	Colchicine Recovered— Spectrophotometric, mg.	Colchicine Recovered— Gravimetric, mg.	Gravimetric Determination of Impurities Recovered from Chromatographic Columns, mg.
50.9	48.3	52.5	3.9
51.4	47.9	51.5	4.0
Mean 51.1	48.1	52.0	3.9
Standard deviation	0.28	1.87	..

[a] Obtained from Mallinckrodt Chemical Works.

DISCUSSION

The use of Struve's color reaction in detecting colchicine for the completion of extraction and elution gives excellent results. Since the color reaction consists of three phases, it is possible to eliminate similarly reacting compounds. This method is applicable only to solutions containing colchicine in high concentration, or their residues when evaporated to dryness.

After removal of fixed oil and fatty substances with petroleum benzene, the colchicine was extracted with ethyl acetate containing 1 ml. of ethyl alcohol. The ethyl acetate was chosen because it extracts a smaller amount of resinous substances than does alcohol. The 1 ml. of ethyl alcohol was added to the ethyl acetate to prevent its hydrolysis. These two steps of extraction were used in order that the separation and purification, carried out solely on chromatographic columns, could produce a crude extract containing fewer interfering impurities during elution.

The crude extract contains a certain amount of resinous substances which are insoluble in chloroform and benzene, but soluble in methyl alcohol. However, some of the extract is also insoluble in the alcohol; consequently, the order of transferring the solution from the extraction flask to the column was by means of methyl alcohol followed by benzene. Alumina, used by Ashley and Harris (12) retained these, but it could not be used for the total purification, as it would not hold most of the nonresinous impurities. A second column, in which the adsorbent was silicic acid, removed these impurities.

The two adsorbents in different columns gave improved results. Alumina retained the resinous substances and some impurities; the colchicine being in the lowest band was eluted first. This eluate obtained from the first separation was easily processed and passed through the silicic acid column.

The Hyflo-Supercel used at the top and bottom of the alumina column serves only as a filter, since it does not adsorb colchicine.

An advantage of this method is that it does not require continuous care, and the many manual manipulations of other methods: e. g., the official N. F. IX method involves shaking with chloroform, causing emulsification of liquid if used too vigorously, too long, or too often; and repeated filtration is required to obtain clear solutions.

Differences obtained in the results from the gravimetric and spectrophotometric methods (Table I) indicate that there are colorless impurities remaining in the solutions, but they do not interfere with the spectrophotometric determination. The spectrophotometric results were always lower (Table IV) than the ones obtained by the gravimetric method, indicating that the impurities in solution did not absorb the transmitted light. A statistical comparison of the determinations presented in Table II indicates that their means are significantly different ($p = >.005$) and greater stress should be placed on results obtained by the spectrophotometric method.

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A Study of Natural Sponge as a Disintegrating Agent in Compressed Tablets*

By ROBERT C. CRISAFI† and CHARLES H. BECKER‡

Natural sponge (*Hippiospongia lachne*, de Laubenfels) has been studied as a disintegrating agent in compressed tablets. A brief description of the procedures used for cleansing, bleaching, and powdering the sponge prior to its incorporation in tablet formulas has been given. The disintegrating ability of powdered sponge in the formulas studied was found to be superior to that of cornstarch for tablets containing lactose, calcium gluconate, sulfadiazine, aluminum hydroxide, bismuth subnitrate, and sodium bicarbonate. Combinations of powdered sponge added during the granulation process and dried cornstarch added just before compression were more efficient for disintegration than powdered sponge alone.

IN A PREVIOUS PAPER (1), Gross and Becker showed that powdered sponge compared favorably with other disintegrating agents commonly employed in tablets. Since their work was limited to lactose tablets and a single percentage of sponge, it was the purpose of the present investigation to make a more detailed study of this agent.

For this investigation sheep's wool sponge (*Hippiospongia lachne*, de Laubenfels) was used because of its availability and commercial importance.

EXPERIMENTAL

The sponge used in this study was in the form of clippings which were trimmed from the sponges in the process of preparing them for market. These clippings contained large proportions of sand and shell as well as other foreign material. Because of the large amount of foreign material and the dark yellowish-brown color of the sponge, it was obvious that a thorough cleansing and decolorization was necessary before it could be used in a tablet formula. Preparation of the sponge for use as a disintegrating agent was accomplished in three steps, cleansing, bleaching, and powdering.

Cleansing of Sponge.—The sand and most of the other foreign material were easily removed from the sponge by washing and hand picking. Following this initial cleansing treatment, the sponge was found to still contain minute pieces of shell embedded within the fibrous network. These calcareous particles were removed by a second wash in a 2% solution of hydrochloric acid.

Bleaching of Sponge.—To decolorize the sponge, the cleansed material was first treated with a 1% solution of potassium permanganate. The sponge was next washed in an acidified solution of sodium bisulfite until the color had been sufficiently bleached. After rinsing in several changes of fresh water, the sponge was dried and prepared for grinding.

Powdering of Sponge.—The sponge was ground in a Wiley mill using two screens, 2 mm. and 1 mm., respectively. The second milling was collected and stored for use in tablet formulas.

Absorption Study.—The mechanism through which powdered sponge and cornstarch accomplish their disintegrating action is most likely the same. Both agents swell when in contact with water and as a result are capable of rupturing tablets in which they are incorporated. Because absorption is a chief factor in this action, tests were conducted on powdered sponge and cornstarch to determine the comparative rate at which this absorption takes place. The moisture conditions were controlled through the use of constant humidity chambers. The materials were first dried to constant weight at 110° and then placed in each humidity chamber for a twenty-four hour period. The weight gain was calculated by reweighing the sample following this twenty-four hour interval and was recorded as per cent of sample weight. Results of these tests are reported in Table I.

TABLE I.—MOISTURE ABSORBED BY POWDERED SPONGE AND CORNSTARCH AT VARIOUS RELATIVE HUMIDITIES

Relative Humidity at 27°, %	Water Absorbed After 24 Hours	
	Powdered Sponge, %	Cornstarch, %
30	9.22	8.13
45	12.21	10.33
60	15.43	12.69
70	17.65	14.14
80	21.36	16.12
95	29.51	20.61

Preparation of Tablets.—The tablet formulas used in this study are listed in Table II. Tablet batches were based on lots of 2,000 tablets for each formula. The medicinal ingredients selected as the active components of the tablets were represented by drugs of variable solubilities. As a control, lactose replaced all of the active medicinal agent in the tablet.

Four series of tablet formulas were prepared. The first, signified by the letter *A* in the batch number, contained 5% powdered sponge added during the process of granulation. The second series,

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Abstracted in part from a dissertation presented to the Graduate Council of the University of Florida by Robert C. Crisafi in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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‡ Professor of Pharmacy, University of Florida, Gainesville.

signified by the letter *B* in the batch number, contained 10% cornstarch which was also added during the granulation process. A third series was made up of the same granulations as series *A* with an additional 2% dried cornstarch added to the dry granules just prior to compression; these formulas are signified by the letter *C* in the batch number. The amount of cornstarch added to this series was intentionally limited since the purpose of these formulations was not to determine the efficacy of cornstarch as a disintegrant, but rather to determine whether or not it would have any effect on the disintegrating ability of the powdered sponge which had been incorporated during the wet granulation. The formulas of the final series, signified by the letter *D* in the batch number, contained the same granulations as series *B* with an additional 2% dried cornstarch added to the dry granulation. Tablets in this last series were prepared and tested in order to obtain a fair evaluation of the data on a comparative basis.

Syrup U. S. P. was used as the binder in the preparation of all granulations. This agent resulted in production of very fine granules. For lubrication, 2% magnesium stearate based on the weight of the finished tablet was used in all formulations. A Stokes rotary, Model B-2, tablet machine was used to compress the completed granulations. The tablets were prepared with standard concave $^{9}/_{16}$-inch punches and dies.

To prepare the granulations, the powdered materials were placed in a Stokes mixer and allowed to mix for one hour. The resultant mixture was sieved through a No. 40 mesh screen.

In order that the percentage of active ingredient and disintegrator remained constant for each series of formulas, it was necessary to predetermine the proportion of binding agent needed for each granulation. This was accomplished through a preliminary experiment on a small amount of each formula. With the proportion of granulating agent determined for the actual granulation process, the adjustment of diluent to obtain a specific tablet weight was calculated.

The thoroughly mixed powders were put in a Readco Dough Type Mixer and the granulating solution was slowly added with constant mixing. The moist mass was granulated by passing it through a No. 8 mesh screen. The coarse granules were spread in thin layers on paper-covered trays and allowed to dry in a circulating hot-air oven at 60°. The time required for drying varied with the nature of the granulation; however, as a rule the time ranged between eight to ten hours. Following the drying period, the granulation was again screened, this time through a No. 14 mesh sieve.

For those granulations that had a tendency to produce excessive "fines" when completely dried, it was necessary to screen the coarse granulation when it was about $^{3}/_{4}$ or $^{2}/_{3}$ dry. After screening a partially dried granulation, it was placed back into the oven and allowed to completely dry with a minimum of fines.

Lubrication of the granules was carried out immediately prior to compression. The lubricant was forced through a No. 80 mesh screen before being added to the granulation.

Measurements of Hardness and Disintegration Time.—To measure the comparative hardness of the tablets prepared in this investigation, the Monsanto Hardness Tester was used. Reported values are averages of five determinations and are stated to the nearest 0.5 Kg. Since tablet hardness has an influence on the rate of disintegration, an attempt was made to maintain the tablet hardness

TABLE II.—TABLET FORMULAS[a,b]

Batch No.	Active Ingredient, %	Binder Syrup U. S. P., %	Disintegrant Sponge, %	Disintegrant Starch, %	Tablet Weight,[c] mg.
I-*A*	78.8 Lactose	14.2	5	..	375
I-*B*	72.7 Lactose	15.3	..	10	375
I-*C*	78.8 Lactose	14.2	5	2	383
I-*D*	72.7 Lactose	15.3	..	12	420
II-*A*	50.0 Bismuth Subnitrate	16.5	5	..	375
II-*B*	50.0 Bismuth Subnitrate	17.4	..	10	375
II-*C*	50.0 Bismuth Subnitrate	16.5	5	2	383
II-*D*	50.0 Bismuth Subnitrate	17.4	..	12	420
III-*A*	50.0 Sulfadiazine	18.0	5	..	350
III-*B*	50.0 Sulfadiazine	21.3	..	10	350
III-*C*	50.0 Sulfadiazine	18.0	5	2	357
III-*D*	50.0 Sulfadiazine	21.3	..	12	392
IV-*A*	50.0 Aluminum Hydroxide	24.3	5	..	400
IV-*B*	50.0 Aluminum Hydroxide	25.0	..	10	400
IV-*C*	50.0 Aluminum Hydroxide	24.3	5	2	408
IV-*D*	50.0 Aluminum Hydroxide	25.0	..	12	448
V-*A*	30.0 Calcium Gluconate	13.6	5	..	400
V-*B*	30.0 Calcium Gluconate	13.5	..	10	400
V-*C*	30.0 Calcium Gluconate	13.6	5	2	408
V-*D*	30.0 Calcium Gluconate	13.5	..	12	448
VI-*A*	45.0 Sodium Bicarbonate	12.1	5	..	350
VI-*B*	45.0 Sodium Bicarbonate	13.7	..	10	350
VI-*C*	45.0 Sodium Bicarbonate	12.1	5	2	357
VI-*D*	45.0 Sodium Bicarbonate	13.7	..	12	392

[a] All percentages of series *A* and *B* were based on the weight of the finished tablet. In series *C* and *D* an additional 2% dried cornstarch was added to the tablet weight of those formulas in series *A* and *B*, respectively.
[b] The remaining weight of the tablet was furnished by the diluent and lubricant.
[c] The weight variation of the tablets was within ±5% of the stated weight.

within a range of 6.0 to 7.5 Kg. Tablets in this hardness range were found to be resistant to breaking and chipping when subjected to friability tests simulating the most extreme handling conditions. In addition, it was felt that a hard tablet would better reveal the disintegration ability of the agents studied.

The disintegration tests were conducted in accordance with the U. S. P. directions (2). A tablet was considered as being disintegrated when it had completely passed through the screen of the basket-rack assembly. Three determinations were made on all tablets tested and the average time was calculated and recorded in minutes.

The average disintegration time as well as the average hardness of the various tablet batches are shown in Table III.

TABLE III.—AVERAGE HARDNESS AND DISINTEGRATION TIME OF TABLETS MEASURED IMMEDIATELY AFTER COMPRESSION

Batch No.[a]	Hardness, Kg.	Disintegration Time, Min.
I-*A*	6.0	3.6
I-*B*	6.5	21.0
I-*C*	7.5	0.9
I-*D*	7.0	19.7
II-*A*	7.0	23.2
II-*B*	6.0	73.9
II-*C*	7.5	4.0
II-*D*	7.5	71.2
III-*A*	6.5	6.3
III-*B*	6.5	98.5
III-*C*	7.0	1.5
III-*D*	7.0	94.4
IV-*A*	7.0	6.1
IV-*B*	6.5	88.5
IV-*C*	6.0	0.7
IV-*D*	6.5	84.3
V-*A*	6.5	13.8
V-*B*	6.0	21.1
V-*C*	7.5	2.3
V-*D*	7.0	58.7
VI-*A*	6.5	2.1
VI-*B*	7.0	20.4
VI-*C*	7.0	0.5
VI-*D*	7.0	19.0

[a] Key to Numerals.—The numeral represents the medicament used in the formulation; I—lactose, II—bismuth subnitrate, III—sulfadiazine, IV—aluminum hydroxide, V—calcium gluconate, VI—sodium bicarbonate.

Key to Letters.—The letter used in conjunction with the numeral indicates the disintegrant or combination of disintegrants used in the formula: *A*—5% powdered sponge added during the wet granulation; *B*—10% cornstarch added during the wet granulation; *C*—2% dried starch added to the dry granulations of series *A*; *D*—2% dried starch added to the dry granulations of series *B*.

DISCUSSION AND CONCLUSIONS

The effectiveness of powdered sponge as a disintegrating agent lies in its ability to absorb liquids. Studies conducted to determine the absorption characteristics of powdered sponge and cornstarch indicate, as shown in Table I, that the moisture absorbed by these agents increased with increased relative humidities. The increase in moisture content by each agent may be illustrated by an S-shaped curve; the curve being more pronounced for powdered sponge, indicating a greater capacity for absorption by this agent.

The mechanism through which sponge absorbs water and other liquids may be attributed to a combination of adhesive and cohesive forces. First, due to adhesion, there is a wetting of the entire surface of the sponge creating an enormous liquid surface. The surface tension effect then tends to reduce this surface to a minimum value through the movement of the liquid into myriads of minute interstitial spaces and capillaries of the sponge skeleton. This causes the sponge particles to swell to a wet volume much greater than their dry volume resulting in an effective disintegrant.

A careful study of the data in Table III reveals two important facts pertaining to the efficacy of powdered sponge as a disintegrating agent for compressed tablets. First, it is noted that tablets in series *A* containing 5% powdered sponge as the disintegrant, disintegrated in all cases more quickly than those tablets in series *B* which possessed 10% cornstarch as the disintegrating agent. Secondly, and of particular interest, is the fact that when an additional 2% dried starch was added to the dry granulation of series *A*, the time required for disintegration of the tablets was markedly reduced Although it may seem that the additional 2% dried cornstarch in tablets of series *C* was totally responsible for the increased rate of the disintegration, this is found not to be true when the disintegration times of the four series of tablets are compared. In making this comparison, it is noted that the reduction in disintegration time for tablets containing an additional 2% dried starch was more pronounced for tablets having powdered sponge as the incorporated disintegrant than for those having 10% cornstarch. This is especially true for those formulas in group II and V. The dried cornstarch, therefore, in addition to serving as a disintegrant *per se*, exhibited a potentiating effect on the disintegrating action of the powdered sponge. This may be explained by taking into consideration the structure of the finished tablets. In formulas of series *A*, the powdered sponge is intimately mixed throughout the tablet granules thereby necessitating intergranular absorption of the liquid media before swelling of the sponge particles takes place. However, when the 2% cornstarch was added to the dry granulation just prior to compression, since it was located around the outside of the granules rather than within the granules, it served to reduce the time required for the disintegration fluid to seep through the outer layers of the tablet granules. This allowed the sponge particles, especially in the granules not occupying the outermost layers of the tablet, to have a more ready access to the disintegration fluid, and, as a result, to decrease the time required for liquid absorption to take place. This in turn increased the rate of swelling of the sponge particles and likewise the disintegration of the tablet. When used in this manner, the starch itself, although acting to some extent as a contributing disintegrant, served principally as an activator to disintegration by hastening the action of the powdered sponge.

The results in Table III also indicate, as has been noted by several other investigators, that the medicament present has a decided influence on disintegration.

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The Detection of 1-Ethinyl Cyclohexyl Carbamate in Toxicological Specimens and its Separation from a Mixture of Other Drugs

By GEORGE R. NAKAMURA

Three different solvent mixtures are presented for the separation of Valamin in combination with some common pharmaceutical compounds, principally, bromural and morphine. Rf's of Valamin and some drugs and their respective colors on the chromatogram are described for three different solvent mixtures. Color tests for Valamin with concentrated sulfuric acid and other reagents using sulfuric acid as a solvent are described.

THE COMMON USE of 1-ethinyl cyclohexyl carbamate, hereafter indicated in this paper as Valamin,[1] as a sedative is acknowledged today; and in a number of cases studied in this laboratory, the drug has been taken by individuals in an attempt to commit suicide. In a few cases, gastric washings contained a mixture of Valamin and bromisovalum (isobromovalerylurea).

Valamin seems to be rapidly metabolized by the body as suggested by the work of Swanson, *et al.* (1), on dogs. We have not been able to recover any Valamin in tissue, blood, and urine specimens obtained from autopsy cases. Bromisovalum was traced in the liver and in the kidney in cases where stomach washings contained large amounts of Valamin and bromisovalum.

Since Valamin shows extremely low ultraviolet and infrared absorption and no fluorescence activity, the detection of the drug was carried out by means of color tests and chromatography.

The precipitation method with ammoniacal silver nitrate of Langecker, *et al.* (2), proved useful but lacked specificity and sensitivity. Other confirmatory tests were sought. Various reagents commonly employed in the toxicology laboratory were tested and our preliminary search indicated that concentrated sulfuric acid, which produces a vivid orange-red color with Valamin, could serve as a fairly sensitive reagent. Comparatively few pharmaceutical compounds react with concentrated sulfuric acid to produce this characteristic color. Some reagents using H_2SO_4 as a solvent were tested with varied results.

EXPERIMENTAL

For the separation of Valamin from bromural and other drugs, paper chromatography was applied. It was apparent from the start that to seek a "universal solvent mixture" to resolve at least a dozen of the drugs which are routinely analyzed in this laboratory would prove futile. An attempt was made to separate some of these compounds which would appear in the basic chloroform extracts, principally morphine, meperidine and bromisovalum.

Ammoniacal silver nitrate spray (3) was employed not only to develop spots and to determine their mobility in the solvent mixtures, but also to differentiate the various drugs by colors.

Extraction.—To 10 Gm. amounts of tissue, gastric contents, or blood, 10 ml. of water was added, and the mixture was homogenized in a blender. The pH of the resulting homogenate was adjusted to 4–5 and the homogenate was heated in a water bath at 100° for thirty minutes. After centrifugation, the supernatant was collected, and the pH was adjusted to 8 with ammonium hydroxide. It was then shaken twice with 50 ml. of chloroform and the aqueous layer was discarded. The chloroform extract was collected and washed with phosphate buffer, pH 7.6. The chloroform layer was filtered through filter paper containing Celite,[2] then evaporated gently to dryness in the steam bath.

Ten milliliters of a urine sample was extracted with chloroform at pH 8.0 without prior steam heating. The chloroform extract was treated as above with Celite and filtered. The filtrate was evaporated to dryness.

Chromatography.—A cylindrical tank 46 cm. in height and 22 cm. in diameter fitted with a ground glass lid was employed for ascending chromatography. This tank accommodates a 44 x 48-cm. sheet of Whatman No. 1 filter paper, rolled and stapled in such a manner that the edges do not contact each other.

The paper was spotted with extract residue containing not less than 50 μg. of Valamin on a base line 3 cm. from the bottom of the paper. The standards listed in Table I were dissolved in ethanol and applied as 50-microgram spots on the same base line. The ascending chromatogram was allowed to develop for eighteen hours at room temperature. The paper was dried, then mist-sprayed with 0.5% aqueous solution of sodium carbonate and dried again. Then the sheet was sprayed with 2% silver nitrate in 10% ammonia water and was dried in direct sunlight to produce a clear color development. A photoflood bulb, about 250 watts, could be used with the same effectiveness.

* Received October 1, 1957, from the Department of Chemistry, 406th Medical General Laboratory, APO 343, San Francisco, Calif.

[1] This drug is available commercially in Japan as "Valamin," a product of Schering in Germany, and the same drug manufactured by Eli Lilly and Co., Indianapolis 6, Ind., is called "Valmid." Inasmuch as the use of "Valmin" is more common in Japan, it was employed for this investigation.

[2] Johns-Manville.

To acidify the atmosphere of the chamber, an evaporating dish containing glacial acetic acid was placed on the bottom of the tank. The paper was sprayed with acetic acid immediately prior to introduction into the tank.

Color Test.—A small amount of residue was placed on a white porcelain plate and a drop of color reagent was added. The color development was observed for a one minute period. Any gradual change of color was noted. Then the plate was warmed on a hot plate and any further change in the color was observed.

RESULTS

The results of chromatographic separation of Valamin and eight other drugs in three different solvent mixtures are presented in Table I. Color test results with concentrated sulfuric acid and reagents (4) using sulfuric acid as a solvent are shown in Table II.

TABLE I.—TYPICAL R_f VALUES OF VALAMIN AND SOME COMMON DRUGS USING THREE DIFFERENT SOLVENT MIXTURES

Drug	Color	Isoamyl Alcohol Paper Impregnated with Glacial Acetic Acid	Cyclohexane Paper Impregnated with Glacial Acetic Acid	Isoamyl Alcohol (Saturated with Conc. NH_4OH), Conc. Ammonium Hydroxide, 3:1 v/v
Valamin	Yellow	0.69	0.65	0.22
Bromisovalum	Pink-Red	0.65	0.69	0.78
Morphine	Blue-Black	0.42	0.22	0.80
Dimenhydrinate	Violet	0.58	0.29	0.25
Benadryl	Violet	0.58	0	0.21
Meperidine	Purple	0.49	0.19	0.24
Codeine	Brown	0.43	0.21	0.75
Chloroquin	Blue-Green	0.15	0.27	0.24
Chloropromazine	Violet	0.59	0.19	0.65

TABLE II.—COLOR REACTIONS OF VALAMIN IN CONCENTRATED SULFURIC ACID AND SOME REAGENTS (4) USING SULFURIC ACID AS A SOLVENT

Reagent	Color	After Heating
H_2SO_4, conc.	Orange	Orange to Red
Mecke	Yellow	Brown
Marquis	Brown	Brown
Froede	Cherry-Red to Blue	Blue to Green
Rosenthaler	Red	Brown
Erdman	Colorless	Colorless
Mandelin (Vanallin-H_2SO_4)	Brick-Red to dull-Orange	Brown
Dimethyl amino-benzaldehyde	Orange-Red	Blood-Red
Molisch	Brick-Red	Brown
Phenol-sulfuric acid	Yellow	Orange

DISCUSSION

In a number of cases in which several hundred-milligram quantities of Valamin were detected in the gastric contents, no substances which gave positive reactions with sulfuric acid were located in the chloroform extracts of urine and blood specimens or in autopsy tissues.

It is assumed, at the present, that Valamin is metabolized very rapidly in man through the destruction of the ethinyl group. Experiments with rat tissue slices and on dog blood levels bear out the metabolic lability of this structural group (5).

While very few common pharmaceutical compounds yield a red color in reaction with sulfuric acid, drugs such as chloropromazine and dimenhydrinate which may occur in the basic chloroform extract should be eliminated as possibilities. Many of the organic bases such as morphine, codeine, heroin, meperidine, ephedrine, amphetamines, methyl parafynol, priscoline, brucine, and chloroquin do not form a red complex with sulfuric acid.

Generally barbiturates and ureides do not react with the acid to form colors but hexobarbital and cyclobarbital do produce an orange-brown to reddish-brown color.

For the chromatography procedure, many laboratory alcohols were tested in various proportions with water in both acidic and basic media to obtain the best possible separation of Valamin from bromisovalum and morphine. Alcohols which were empirically tested in various mixtures were methanol, ethanol, *n*-propanol, iso-propanol, *n*-butanol, iso-butanol, *n*-amyl alcohol, and iso-amyl alcohol. Other solvent mixtures contained acetone, chloroform, ethyl acetate, pyridine, propylene glycol, cyclohexane, benzene, and collidine. Schmall, *et al.* (6), introduced an elegant paper chromatography method by which organic bases were separated on a multibuffered paper. This laboratory has found this technique useful for the separation of morphine and codeine, but Valamin could not be immobilized in any of the buffer zones above pH 2.0.

It was discovered that impregnating the paper with glacial acetic acid prior to irrigation provided clear, compact spots. Merely saturating a paper in an atmosphere of acetic acid, even for a twelve-hour period, was not comparable to the effect produced by actually wetting the paper with the acid.

A mixture of Valamin and bromisovalum proved to be very difficult to separate as the two compounds seem to possess identical mobility patterns in nearly all of the solvent preparations tried except in a two-phase system of isoamyl alcohol and ammonia. Three solvent mixtures which gave the best separa-

tion of Valamin with respect to eight other drugs are presented in Table I. The R_f values presented in the table do not imply that the various substances, including Valamin, listed herein can be identified by these values alone, inasmuch as the R_f differences are so small between certain compounds.

Fortunately bromisovalum and Valamin each reacted to ammoniacal silver nitrate with distinct colors (see Table I). Identification of drugs by the color of their spots on a chromatogram proved to be another useful criterion.

The color development of Valamin with concentrated sulfuric acid is extremely sensitive but not specific, however the results obtained from the chromatography procedure were of aid in the elimination of the possible presence of the few compounds which produce colors similar to Valamin in sulfuric acid.

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A Comparison of the Pharmacological Properties of Two Nitrated Cyclohexanols*

By WILLIAM J. FLEMING,† EDWARD J. ROWE, and DONALD B. MEYERS

The nitrate esters of two isomeric cyclohexanols, *meso*-inositol and scyllitol, were prepared and their pharmacological effects studied. The compounds exhibited the typical organic polynitrate action of depressing smooth muscle when tested on various isolated tissues such as the rabbit intestine, guinea pig ileum, and rat and rabbit lungs. A typical vasodepressor action was obtained from anesthetized cats and rabbits. The acute toxicity of the nitrates was also determined. The results of this study showed the *meso*-inositol nitrate to be the more active of the two compounds in depressing smooth muscle of the organs studied. No species variation was noted.

SINCE THE INTRODUCTION of glyceryl trinitrate as a medicament nearly one hundred years ago, various organic polynitrates have been utilized as smooth muscle relaxants; however, relatively little is known regarding their mechanism of action. Since the compounds exhibit activity similar to the metallic and organic nitrites, it was postulated by Hay (1) and substantiated by others (2, 3) that the organic nitrates were converted to nitrite ion in the blood and exerted their physiological actions by this mechanism.

More recently, Krantz and co-workers, in a series of papers (4–7), offer evidence that the organic polynitrates act as intact molecules and not through hydrolysis and reduction to nitrite. This assumption is also held by Marshall (8).

Thus far, little has been done in correlating the spatial configuration necessary to obtain maximum depressor activity of the organic nitrates. It is known that methyl nitrate has little depressor properties while most of the active members of the series are derived from polyalcohols. However, Marshall found that the arrangement of the nitrate groups on a series of isomeric hexahydric alcohols did not appear to influence the activity of the compounds (8). In this study, a comparison of the relative potency of two isomeric nitrated cyclohexanols was undertaken.

* Received April 16, 1956, from Butler University College of Pharmacy, Indianapolis, Ind.
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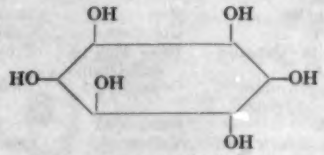
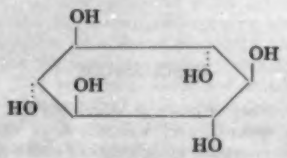
EXPERIMENTAL

Preparation of the Nitrated Products of *meso*-Inositol and Scyllitol.—The spatial configurations of the two isomers of inositol which were used in this study are given below (9).



meso-Inositol



Scyllitol

Inositol Nitrate.—*meso*-Inositol nitrate was prepared by dissolving 5 Gm. (0.028 moles) of anhydrous *meso*-inositol in 10 ml. (0.22 moles) of cold, fuming nitric acid. After complete solution was obtained, 10 ml. of fuming sulfuric acid was slowly added with constant stirring. During this time a white, magma-like mass formed. The temperature maintained throughout the synthesis was 0–5°. The precipitate was diluted with cold distilled water, filtered, washed thoroughly with cold water, and dried under a carbon dioxide atmosphere.

The product, when recrystallized two times from a 2:1 v/v mixture of 95% ethanol and water and two times from benzene, melted at 119–121°[1] which is in agreement with that recorded for the hexanitrate. A yield of 31.6% was obtained.

meso-Inositol nitrate is a fine, white, crystalline powder which decomposes readily in the dry form when exposed to the atmosphere at room temperature. It is insoluble in water but is soluble in alcohol, propylene glycol, acetic anhydride, and warm benzene.

Anal.—Calcd. for $C_6H_8N_6O_{18}$: N, 18.67. Found: N, 17.48 (Kjeldahl).

Scyllitol Nitrate.—The nitrated product of scyllitol was prepared by adding, with stirring, 4 Gm (0.022 moles) of scyllitol to 10 ml. (0.22 moles) of fuming nitric acid. Upon standing, a white, magma-like mass formed which did not dissolve in the acid. Fuming sulfuric acid, 10 ml., was slowly added with constant stirring. The temperature was maintained at 0–5° throughout the procedure. The precipitate was diluted with ice water, filtered, washed thoroughly with cold water, and dried under a carbon dioxide atmosphere.

The final product is a dense, white powder which melted at 123–125° with decomposition. A yield of 90.75% was obtained. It is insoluble in water and the common organic solvents.

Anal.—Calcd. for $C_6H_8N_6O_{18}$: C, 16.01; H, 1.33; N, 18.66. Found: C, 18.05; H, 1.57; N, 15.84 (Kjeldahl).

PHARMACOLOGY

Since the organic polynitrates exhibit relaxing actions on many organs containing smooth muscle, a pharmacological comparison of the two compounds was made by studying their relative effects on the vascular system, small intestine, uterine muscle, trachial chain, and isolated lung.

In all studies except that of acute toxicity, the compounds were administered in a propylene glycol vehicle. *meso*-Inositol nitrate was soluble in a concentration of 10 mg./ml., while the scyllitol nitrate formed a uniform suspension. Acute toxicity studies of both drugs were carried out with the compounds suspended in a 0.5% methylcellulose solution.

PERFUSION STUDIES

Isolated Rabbit Intestine.—Randomly selected segments of small intestine from non-fasting, albino rabbits were isolated and suspended in water baths containing aerated Tyrode's solution at a temperature of 37–38°. The volume of the bath was 100 ml. throughout the study. The free ends of the segments were attached to lever-arms for kymograph recording.

The results of this experiment showed that *meso*-inosotol nitrate, in doses of 0.5–1.0 mg., was active in lowering both the tone and amplitude of intestinal muscle while comparable concentrations of scyllitol nitrate exhibited little activity.

The vehicle, propylene glycol, caused an immediate slight fall in tone which was followed by an increase in tone and a decrease in amplitude when administered in doses of 3 ml.

Antagonism Against Histamine Induced Spasm of Isolated Guinea Pig Ileum.—Ileal segments of nonfasting guinea pigs were isolated and suspended in a water bath containing aerated Locke-Ringer's solution at a temperature of 37–38°. The bath volume was 100 ml. throughout the experiment. The system was organized for kymograph recording in the usual manner.

After tracings of normal ileal movements were obtained, 0.1 ml. of histamine acid phosphate, 1:1000, was added to the preparation to produce an immediate ileal spasm. The drug was left in contact with the tissue for two and one-half minutes. The segment was then washed and the contractions allowed to return to normal. The same procedure was followed in subsequent trials, with the alteration that thirty seconds after the dose of histamine acid phosphate, *meso*-inositol nitrate or scyllitol nitrate was added in varying concentrations. Their effects of alleviating the spastic ileum were noted over a time interval of two minutes.

It was determined by this study that *meso*-inositol nitrate was 1.46 times as active as scyllitol nitrate in doses of 1.5 mg., and 1.99 times as active in doses of 1.0 mg., in relaxing the spastic ileum after the addition of histamine.

Guinea Pig Tracheal Chain.—The tracheae of two albino, female guinea pigs were used to prepare a chain according to the method of Castillo and DeBeer (10). The chain was suspended in aerated Van Dyke-Hastings solution modified by the addition of 0.05% dextrose. The bath was maintained at a temperature of 37–38° and a volume of 100 ml. throughout the experiment.

Histamine acid phosphate, 0.5 mg., was added to the bath to cause a contraction of the chain. Neither of the compounds under study, in doses up to 3 mg., produced any antagonism to histamine induced tracheal spasm when administered before or after the histamine.

Isolated Guinea Pig Uterus.—Uterine segments of nongravid guinea pigs were obtained and suspended in aerated Locke-Ringer's solution at a temperature of 37–38°. The bath volume was 100 ml.

Doses as high as 3 mg. of either *meso*-inositol nitrate or scyllitol nitrate had no activity in relaxing the tissue or antagonizing contractions caused by posterior pituitary, U. S. P., in concentrations of 1×10^{-3} units/ml.

Isolated Lung Perfusion.—The lungs and trachea were removed from a rabbit or rat and placed on a T-type cannula. Modified Locke-Ringer's solution was then perfused through the lung at a positive, constant pressure and a temperature of 37–38°. After expressing the residual air in the lungs through the sidearm of the cannula, the drugs were injected into the perfusion fluid at a rate of 1 ml./min. and

[1] The melting points were determined with a Fisher-Johns apparatus.

their actions in altering the rate of outflow were noted.

It was found that *meso*-inositol nitrate in doses of 5 mg. and 10 mg. was active in increasing the lung outflow in the rat, but the results were not conclusive in the rabbit study. Scyllitol nitrate was somewhat active in increasing outflow in both the rat and rabbit; however, the compound was not as potent as its *meso*-inositol isomer in duration of action or outflow when compared in equal doses on the isolated rat lung.

BLOOD PRESSURE STUDIES

Anesthetized Rabbit.—Six albino rabbits of either sex and weighing 3.5–4.1 Kg. were anesthetized with urethane, 1.5 Gm./Kg., rectally and maintained throughout the experiment with intravenous injections of secobarbital sodium as needed. The animals were prepared for blood pressure and respiration recording by cannulating the right common carotid artery and trachea. All injections were introduced through the marginal ear vein.

Both of the drugs were effective in lowering blood pressure in doses of 5 mg. (Table I). The fall in blood pressure took place within ten seconds after the injection had begun and was accompanied by an increase in the rate and amplitude of respiration. Doses lower than 5 mg. produced rather transient depressions in pressure. The same general responses were obtained after double vagotomy.

Anesthetized Cat.—Three female cats weighing 2.1–2.9 Kg. were anesthetized with phenobarbital sodium, 175 mg./Kg., by intraperitoneal injection. The animals were prepared for blood pressure and respiratory studies in the usual manner. All injections were introduced through the left external jugular vein.

The results of this study are essentially the same as observed in the rabbit and are tabulated in Table I. It can be seen that *meso*-inositol nitrate is the more active of the two compounds from the standpoint of the amount of fall in pressure and duration of action.

TABLE I.—BLOOD PRESSURE STUDY IN THE CAT AND RABBIT

Drug	Dose, mg.	Av.[a] Fall in Systolic B.P., mm. Rabbit	Cat	Av.[a] Duration of Action, min. Rabbit	Cat
m-Inositol Nitrate	5	23	..	8.8	..
Scyllitol Nitrate	5	19	..	5.3	..
m-Inositol Nitrate	10	32	33	9.6	11
Scyllitol Nitrate	10	21	21	6.6	9
m-Inositol Nitrate	15	30	31	5.1	9.5
Scyllitol Nitrate	15	19	22	6.2	7.5

[a] Av. in 6 rabbits and 3 cats.

Acute Toxicity-Intraperitoneal LD_{50}.—Albino mice, weighting 14–20 Gm., were injected intraperitoneally with the drugs suspended in a 0.5% methyl cellulose solution. The test animals were placed in cages by dose and observed over a period of 72 hours.

The majority of mortalities occurred after 24 hours and before 48 hours. The only apparent toxic sign was a cyanosis seen in the tail four hours after injection.

The LD_{50} and standard error of the compounds was calculated by the method of Bliss (11). The LD_{50} of *meso*-inositol nitrate was found to be 0.957 ± 0.057 Gm./Kg. The LD_{50} of scyllitol nitrate was computed to be 1.267 ± 0.088 Gm./Kg.

DISCUSSION

The data obtained from the pharmacological comparison of the nitrated products of *meso*-inositol and scyllitol indicates that *meso*-inositol nitrate is the more active of the two compounds in relaxing the smooth muscle of the various organs studied from the standpoint of the amount of activity and duration of action.

In comparing the relative potency of the two drugs, several factors should be considered: spatial relationships, solubility, and the degree of nitration obtained.

It can be seen from the structural configurations that *meso*-inositol has three adjoining hydroxyl groups on the same side of the plane of the cyclohexane ring, while the hydroxyl groups of scyllitol alternate above and below the plane of the ring; and therefore, none of the groups are directly adjacent. As yet, the relationships necessary for the organic polynitrates to exhibit maximum depressor activity is not known, but since methyl nitrate and ethylene glycol dinitrate have little smooth muscle relaxant properties while activity increases with higher members of the series, it may be possible to draw some correlation of activity with the number of adjacent nitrate groups on the molecule.

Secondly, from the previously described physical properties of the two compounds, it is evident that scyllitol nitrate is generally the more insoluble. This factor could well play a part in limiting the activity of the drug by altering its contact and fixing abilities with the smooth muscle tissue.

Although analytical determinations indicate that *meso*-inositol nitrate was more completely nitrated than the scyllitol derivative, the importance of this factor is not known; however, in most cases the pharmacological response produced by *meso*-inositol nitrate could not be obtained with even twice the quantity of scyllitol nitrate.

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Peristaltic-Stimulating and Fecal-Hydrating Properties of Dioctyl Sodium Sulfosuccinate, Danthron, and Cascara Extracts in the Mouse and Rat*

By PAUL M. LISH and KENDRICK W. DUNGAN

Utilizing the criteria of fecal-hydration and peristaltic-stimulation the potency and properties of Colace (dioctyl sodium sulfosuccinate), danthron N. F., and four cascara extracts were investigated in mice and rats. The results suggested that Peristim (Casanthranol), would be the most suitable peristaltic-stimulating agent as an adjunct to Colace therapy. Peristim was approximately 3 times as active as Cascara Sagrada Extract N. F. The peristaltic-stimulating action of Peristim was demonstrated in normal rats, in rats after postoperative intestinal stasis, and after ganglionic blockade. Peristaltic-stimulating properties could not be demonstrated for danthron in rats. Danthron and Peristim showed similar abilities to induce fecal-wetting in mice but this action of danthron, unlike that of Peristim, was strongly inhibited by ganglionic blockade. Colace was approximately 3.5 and 2.2 times as potent as Peristim in producing fecal-wetting in mice and rats respectively. The fecal-hydrating action of Colace was inhibited in the mouse and rat by chlorisondamine and atropine, but not by bile duct cannulation in the rat. As assessed by the mouse fecal-hydrating test, Peristim and Colace were additive while danthron and Colace were not.

THE ORAL ADMINISTRATION of dioctyl sodium sulfosuccinate, Colace[1] to humans has been shown to cause effective fecal softening (1, 2) with otherwise negligible pharmacological effects (3). It has been suggested that in some patients a laxative agent with peristaltic-stimulating action would be a desirable adjunct to Colace therapy (2, 4). The ideal peristaltic stimulant should be effective in spite of a variety of normal or abnormal conditions interfering with the neuronal or muscular function of the intestine. Cascara sagrada or its various extracts have been credited with being among the most suitable of the stimulant laxatives showing a minimum incidence of griping and other side effects (5, 6). A water soluble, bitter free concentrate of the active laxative principles of cascara sagrada was developed by Lee, *et al.*, and this substance, Peristim,[2] was reported to be approximately ten times as potent as whole cascara sagrada in humans (7).

The purpose of the following study was to verify the relative potency of cascara sagrada and Peristim and to examine some characteristics of Peristim, in mice and rats, in an effort to gain information indicative of its utility as an adjunct to Colace therapy in humans. Danthron, N. F. (1-8-dihydroxyanthraquinone, chrysazin) was similarly investigated.

EXPERIMENTAL

Materials.—Peristim and its fractions, the isopropanol soluble Casanthranol B and the isopropanol insoluble residue Casanthranol A, were supplied by S. B. Penick and Company. Danthron was in the form of the micronized powder. Peristim and Casanthranol fractions A and B were administered orally as solutions in distilled water. Cascara sagrada extract[3] and danthron were suspended in 0.5% methylcellulose 400. The suspensions were passed through a hand homogenizer several times before use and stirred vigorously before each dose. Colace was administered as an aqueous solution and it was freely miscible with all agents; in fact, it improved the consistency of the cascara and danthron suspensions. In the synergism experiments Colace was added to the Peristim solution or danthron suspension. The charcoal mix employed in the rat intestinal-propulsion tests was 6% charcoal in 1% methylcellulose 400.

The influence of several drugs upon the peristaltic-stimulating actions or fecal-hydrating properties was determined. The following drugs were chosen because of their ability to block histamine or various levels of the autonomic nervous system: atropine sulfate, chlorisondamine, phenoxybenzamine, meprobamate, and pyrilamine maleate. All these blocking agents were administered subcutaneously and, with the exception of meprobamate, were dissolved in normal saline. Meprobamate was dissolved in 50% polyethylene glycol 400 and normal saline. The albino mice were all males of the Swiss Webster strain weighing between 18 and 25 Gm. Male and female rats of the Hamilton laboratory stock (Wistar and Carworth Farm) weighing between 150 and 250 Gm. were employed.

Peristaltic-Stimulation Methods.—Propulsive rates of a charcoal mix were determined by the method of Macht (8). The charcoal mix was administered via stomach tube in a volume of 5 cc./Kg. Preliminary experiments revealed that the maximum peristaltic-stimulating effect of either cascara sagrada or Peristim occurred at three to

* Received August 29, 1957, from the Mead Johnson Research Laboratories, Evansville, Ind.

[1] Mead Johnson & Company trade name for dioctyl sodium sulfosuccinate.

[2] Mead Johnson & Company trade name for a methanol extract of cascara sagrada bark.

[3] Cascara sagrada extract N. F. used throughout this study.

four hours following oral administration. Therefore, the charcoal mix was administered three hours and fifteen minutes after the drug and the rats were sacrificed forty-five minutes later. Control animals received the vehicle alone.

Peristim and its fractions (Casanthranol A and Casanthranol B) and cascara sagrada extract were compared for their propulsion-stimulating effects by using a self-contained 4-point assay in which there were 30 rats at each dosage level. Peristim was arbitrarily chosen as the standard. The results of the assay were evaluated as outlined by Smith and Vos (9) and Knudsen (10).

The effect of Peristim on the rate of stomach emptying during the interval of three to five hours following its administration was determined by a modification of the method of Reynell (11). Three hours after Peristim administration a solution of phenol red, 5 cc./Kg., was given via stomach tube and the homogenized stomach and contents analyzed for the dye at 0, 30, 90 and 180 minutes. The concentration of dye at zero time less the concentration at the appropriate interval was expressed as per cent of stomach emptying.

Postoperative intestinal stasis, as defined by a decreased propulsive rate of charcoal mix, was induced by opening the abdominal cavity under ether anesthesia and manipulating the contents in the same fashion in all rats. The contribution of the operative procedure toward the decreased propulsive rates observed was not determined. Ether anesthesia alone was found to induce similar effects.

Fecal-Hydration Methods.—The technique utilizing an "all or none" response essentially as described by Miller (12) for mice was used in both mice and rats. Certain modifications suggested by Lou (13) were adopted as follows: (*a*) the animals were observed for a period of two hours prior to dosing and those producing abnormally wet feces were discarded; (*b*) during the test observation period the animals were allowed free access to a special food consisting of equal parts of laboratory chow and tap water. Other modifications adopted involved the withdrawal of food, but not the water, three hours and eighteen hours before dosing the mice and rats, respectively. An attempt was made to achieve uniform hydration of the animals by adjusting the concentration of the fecal-hydrating agent so that upon dosing each animal received a water load, 30 cc./Kg. for the mice and 20 cc./Kg. for the rats. As described by Miller (12) observations were made at appropriate hourly intervals and consisted merely of recording whether or not the feces of the mouse or rat were sufficiently moist to stain the absorbent paper inserted berween the wide mesh bottom of each individual cage. Evaluations were made by the method of Litchfield and Wilcoxon (14). In all cases in which an "effective dose$_{50}$" (ED_{50}) was determined, there were 20 animals at each dosage level and at least 60 animals in each determination.

As a means of demonstrating not only the potency but the speed of onset of the fecal-wetting action, dose response curves were drawn and ED_{50} values determined, from the same mice, at the two-hour, five-hour, and eight-hour periods, (Table II).

In some experiments the abdominal cavity of rats was opened under ether or pentobarbital anesthesia and Colace was deposited, via hypodermic syringe, at various levels of the G.I. tract. Such rats were returned to their individual cages above the absorbent paper and the feces were examined in the usual manner.

RESULTS

Peristaltic-Stimulating Effects—Rats.—In eleven groups of 20–30 rats each, it was found that controls invariably propelled the charcoal mix so that the farthest point was a mean distance of 61 ± 3% of the total length of the intestine. When the stimulated peristalsis produced by the drug caused the farthest point of charcoal to lie between the 65% and 85% level, there was an approximately linear log dose-response relationship. The cecum, which falls at about the 90% level, was a block to charcoal movement and larger doses of drug would simply increase the percentage of animals in which the charcoal had been propelled to the cecum.

The results of the assay, presented in Table I, show that cascara sagrada extract was about 1/3 as potent as Peristim or fraction A. The possibility was considered that the lower activity of fraction B might be due to the absence of some suspected synergistic substance which was possibly present in fraction A. This was not the case since combining the two fractions yielded a mixture possessing only the algebraic sum of the two and not the activity of Peristim. The increased propulsive rates seen in Peristim-treated rats were not due to an increased rate of stomach emptying for tests showed that such animals emptied phenol red from their stomachs at a slower rate than did controls.

Figure 1 shows graphically the ability of Peristim to overcome postoperative intestinal stasis and antagonize the propulsion inhibiting effects of a

TABLE I.—COMPARATIVE ABILITY OF CASCARA SAGRADA EXTRACTS AND DANTHRON TO STIMULATE THE PROPULSIVE RATE OF A CHARCOAL MIXTURE IN RATS

Laxative Agent	Oral Dose, mg./Kg.	Mean % of Travel	Relative Potency ± Std. Error
Controls[a]	Vehicle only	59.4 ± 1.52	
Peristim	220	70.8	
	480	78.5	100
Cascara sagrada extract N. F.	600	70.8	
	1310	79.1	37 ± 3.3
Casanthranol A	220	69.5	
	480	82.0	108 ± 6.5
Casanthranol B	480	64.0	
	1050	77.3	34 ± 2.1
Mixture: 50% Casanthranol A & 50% Casanthranol B	220	67.2	
	480	77.3	80 ± 4.6
Danthron[b]	120	58.3 ± 1.94	
	240	63.5 ± 1.7	0
	480	63.2 ± 2.5	
	960	62.3 ± 1.3	

[a] Mean ± std. error of 60 animals.
[b] Mean ± std. error of 20 animals.

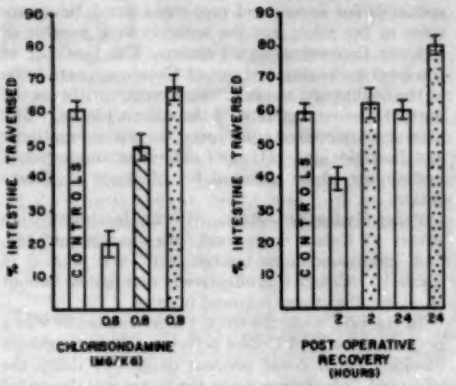

Fig. 1.—Effect of Peristim 240 mg./Kg. (striped area) and 480 mg./Kg. (dotted area) on the decreased propulsive rates induced by a ganglionic blocking agent or abdominal operation under ether anesthesia. Vertical bars indicate standard error of the mean; 20 rats per group.

ganglionic blocking agent. Peristim, in appropriate dosage, was able to decrease or completely overcome the inhibiting effect of chlorisondamine. The stimulating effects of Peristim were therefore considered to be largely independent of functioning intestinal reflexes. Propulsive rates were measured in the danthron-treated rats at intervals varying from forty-five minutes to six hours following drug administration, but there was no evidence of peristaltic stimulation with doses as high as 960 mg./Kg. Colace in doses of 20 mg./Kg. to 100 mg./Kg. showed no significant effect upon propulsive rates. Colace at 200 to 400 mg./Kg. tended to inhibit propulsive rates.

Fecal Hydration—Mice.—A comparison of the ability of six agents to cause excretion of wet feces in mice is shown in Table II. In this test, as in the rat peristaltic-stimulating test, cascara sagrada extract was approximately $^1/_3$ as potent as Peristim. The slower onset of action of the cascara extracts did not permit calculation of an ED_{50} at the two-hour period following drug administration. The onset of action of danthron was somewhat more rapid than that of the cascara extracts but less rapid than that of Colace. Colace was about nine times as potent as cascara sagrada extract and three and one-half times as potent as Peristim. Only in the case of danthron did a dose response curve, at the eight-hour test period, deviate significantly (19/20 probability) from parallelism with the Peristim curve. Comparison of the ED_{50} of these two agents was therefore less meaningful. Between the levels of 40% and 90% effectiveness, danthron was respectively two and one-half times more potent and two and one-fifth times less potent than Peristim.

In an experiment designed to detect addition of drug action (Fig. 2), the dose-effect curve for Peristim obtained from mice treated with $^1/_2$ the ED_{50} of Colace (0.5 Gm./Kg.) had essentially the same slope as that in mice not receiving Colace. A reasonable criterion for addition of effects was therefore that $^1/_2$ the dose of Peristim required to produce an 80% effect in normal mice should produce the full 80% effect in such Colace-treated mice. Figure 2 demonstrates that this was the case. The ED_{50} expected for danthron in identical experiments was 3.1 Gm./Kg. but that found was greater than 8.0

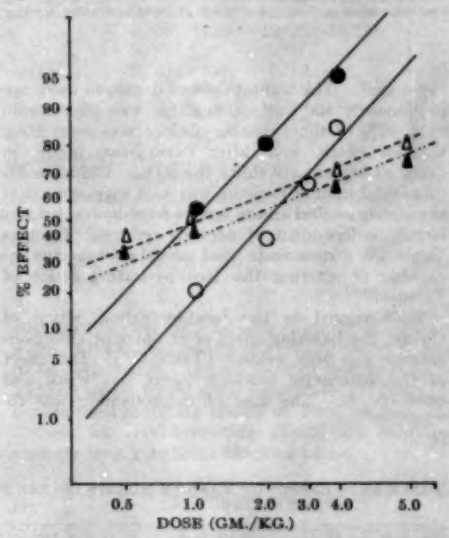

Fig. 2.—Mouse fecal-hydrating test, eight-hour test period. Dose effect lines of: Peristim in normal mice ○—○; Peristim in Colace-treated mice ●—●; danthron in normal mice △—△; danthron in Colace-treated mice ▲---▲.

TABLE II.—POTENCY OF VARIOUS SUBSTANCES, MOUSE FECAL-STAINING METHOD

Drug	ED_{50} Gm./Kg. (19/20 Confidence Limit) Time Post Drug Administration 2 Hr.	5 Hr.	8 Hr.	Slope Function "S" 8 Hr.	Potency Ratio Peristim = 1 (19/20 Confidence limits), 8 Hr.
Peristim	>4.0	2.26 (1.9–2.69)	2.10 (1.6–2.7)	2.0	1.0
Casanthranol A	>4.0	1.76 (1.38–2.25)	1.20 (0.98–1.46)	2.3	1.75 (1.27–2.42)
Casanthranol B	>4.0	1.7 (1.13–2.55)	1.10 (0.97–1.24)	1.7	1.91 (1.44–2.54)
Cascara sagrada	>12.0	5.2 (3.9–7.0)	5.2 (3.9–7.0)	3.2	0.41 (0.29–0.59)
Danthron	5.0 (3.1–8.0)	1.6 (1.1–2.4)	1.2 (0.6–2.4)	7.3	1.75 (0.74–3.64)
Colace	0.61 (0.47–0.8)	0.59 (0.45–0.77)	0.59 (0.45–0.77)	1.8	3.56 (2.6–5.0)

Gm./Kg. Although Colace depressed danthron activity at all dose levels, the depression was not statistically significant.

Ganglionic blockade influenced the ED_{50} of the agents as shown in Table III. The onset of positive responses was not necessarily delayed in the chlorisondamine-treated mice but fewer animals responded. The highest dose of danthron used, approximately six times the ED_{50}, was effective in only 40% of the animals. Colace was completely without effect, even after twenty-two hours, in doses as high as six times the ED_{50}. Chlorisondamine-inhibition of Peristim was mild suggesting that the ability of Peristim to induce fecal-hydration was largely independent of nervous reflexes. Data in Table IV demonstrate that atropine was also incapable of altering the fecal-hydrating action of Peristim.

TABLE III.—EFFECT OF SUBCUTANEOUS CHLORISONDAMINE ADMINISTRATION[a] ON THE FECAL-HYDRATING EFFECTS OF SEVERAL AGENTS IN MICE

Drug	8 Hr. ED_{50} with chlorisondamine, / ED_{50} without chlorisondamine
Peristim	1.7
Casanthranol A	1.7
Casanthranol B	1.8
Danthron	>6.0
Colace	>6.0

[a] Chlorisondamine administered, in a dose of 0.8 mg./Kg. one hour after oral administration of fecal-hydrating agent.

With regard to the fecal-hydrating action of Colace, the blocking effect of atropine or chlorisondamine was very evident (Table IV). The effect of the adrenergic blocking agent was weak and questionable. The dose of meprobamate was responsible for severe and prolonged paralytic symptoms in the mice, but the animals were capable of righting themselves at all times. The blockade of the fecal-hydrating activity of Colace appears to be at the cholinergic smooth muscle receptor sites or the parasympathetic ganglia of the enteric plexus. The hemodynamic effects produced by the sympathetic ganglion blocking action of chlorisondamine would not appear to be essential for blockade of Colace action.

Fecal-Hydration—Rats. — The fecal-hydrating effects of Colace in normal, bile duct-cannulated, and chlorisondamine-treated rats are shown in Table V. Colace was effective in rats in doses about $^1/_5$ as small as those required in mice.

In the rats, as in the mice, chlorisondamine was a potent inhibitor of Colace activity. This ganglionic blocking agent could prevent or greatly delay the effects of Colace even when the latter was deposited in the proximal colon. That a continual source of bile was not necessary for Colace activity was shown in the rats with bile duct cannulae.

An experiment was conducted in which rats received either with or without a standard dose of Colace, dried whole ox bile, pancreatin, or a combination of bile and pancreatin. These substances did not alter the response of the rats to Colace and had no apparent effect when administered alone.

The results of the fecal-hydration studies of Peristim in normal rats appear in Table VI. The attainment of a near maximal response in rats was somewhat slower than in mice but Peristim was about 10 times more effective in rats than in mice.

DISCUSSION

The fecal-hydration test in the mice and the peristaltic-stimulation studies in rats show Peristim to be 2.5 to 3 times as potent as cascara sagrada extract. Since one gram of the extract represents three grams of the whole bark, the experimental results agree well with the clinical impressions (7) that this sub-

TABLE IV.—EFFECT OF VARIOUS AGENTS ON THE FECAL-HYDRATING ACTION OF PERISTIM AND COLACE IN THE MOUSE

Fecal-Hydrating Agent	Drug	mg./Kg.	Time of Administration[a] (Minutes Postfecal-Hydrating Agent)	—Positive Response/No. Tested— 1 Hr.	5 Hr.	8 Hr.	22 Hr.
Peristim, 4.0 Gm./Kg. Oral	H_2O controls	30 ml.	60 (10 ml./Kg.) 120 (10 ml./Kg.) 180 (10 ml./Kg.)	0/20	17/20	20/20	20/20
	Atropine sulfate	12	60 (4 mg./Kg.) 120 (4 mg./Kg.) 180 (4 mg./Kg.)	0/10	8/10	9/10	9/10
	Atropine sulfate	36	60 (12 mg./Kg.) 120 (12 mg./Kg.) 180 (12 mg./Kg.)	0/10	8/10	10/10	10/10
Colace, 1.0 Gm./Kg. Oral	H_2O controls	10 ml.	10	6/10	7/10	7/10	7/10
	Atropine sulfate	1.0	10	3/10	3/10	3/10	5/10
	Atropine sulfate	8.0	10	0/10	0/10	1/10	2/10
	Chlorisondamine	0.1	10	3/10	3/10	3/10	5/10
	Chlorisondamine	0.8	10	0/10	0/10	0/10	0/10
	Phenozybenzamine	1.0	10[b]	8/10	8/10	8/10	8/10
	Phenoxybenzamine	10.0	10[b]	2/10	5/10	5/10	5/10
	Meprobamate	150.0	10	1/10	3/10	3/10	3/10
	Pyrilamine maleate	2.0	10	9/10	9/10	9/10	9/10

[b] Phenoxybenzamine administered 10 minutes before Colace.

stance was about 10 times as potent as whole cascara sagrada.

Although Peristim and danthron appear to have approximately equal ability to produce soft hydrated stools in the mouse, this work clearly indicates that the actions of the two substances are not identical. The dose-response curves markedly deviate from parallelism in the mouse fecal-hydration test; danthron lacks the peristaltic-stimulating effects of Peristim in the rat, and danthron is much more susceptible than Peristim to inhibition by ganglionic block in the mouse.

lionic block or intensive anticholinergic treatment. The results suggest that, in the human, Peristim would be a reliable peristaltic-stimulant even in cases of hypomotility induced by operative procedures, anticholinergic, antispasmodic, or hypotensive therapy.

Colace was demonstrated to be a very effective fecal-hydrating agent in both the mouse and rat. Its action was susceptible to inhibition and the results suggest that, in the human, natural or induced conditions associated with decreased intestinal activity and autonomic neuronal transmission may interfere

TABLE V.—FECAL-HYDRATING ACTION OF COLACE ADMINISTERED AT VARIOUS LEVELS OF THE G.I. TRACT OF THE RAT; THE EFFECT OF BILE DUCT CANNULATION AND GANGLIONIC BLOCK

Condition of Rats[a]	Site of Administration of Colace[b]	Positive Response No. Treated (Hours Post Colace Administration) 1	3	5	8	22
Normal	Oral 25 mg./Kg.	0/20	1/20	1/20	3/20	...
Normal	Oral 50 mg./Kg.	0/20	1/20	3/20	5/20	...
Normal	Oral 100 mg./Kg.	0/20	7/20	12/20	13/20	...
Normal	Oral 200 mg./Kg.	2/20	18/20	18/20	19/20	...
Normal	Jejunum	9/10	...	9/10	...	9/10
Normal	Jejunum (water only)	0/10	...	0/10	...	0/10
Bile duct cannulated (pentobarbital anesthesia)	Jejunum	6/10	...	10/10	....	10/10
Chlorisondamine treated[c]	Jejunum	0/10	...	0/10	0/10	9/10
Normal	Proximal colon	6/8	...	8/8	...	8/8
Normal	Proximal colon (water only)	0/9	...	0/9	...	0/9
Chlorisondamine treated[c]	Proximal colon	0/10	...	0/10	0/10	10/10

[a] Exclusive of operation necessary for Colace administration; this was always done under ether anesthesia unless otherwise specified.
[b] Colace given in dose of 0.2 Gm./Kg. unless otherwise specified.
[c] Subcutaneous administration of 0.8 mg./Kg. at the time of Colace administration.

TABLE VI.—FECAL-HYDRATION TEST IN RATS, EFFECT OF PERISTIM

Oral Dose Peristim, Gm./Kg.	Positive Response/ Total Rats Hr. Post Peristim Admin. 4	5	8	8-hr ED50, Gm./Kg.
0.125	2/20	3/20	8/20	
0.25	1/20	5/20	12/20	0.175
0.5	3/20	8/20	17/20	
1.5	7/20	14/20	19/20	

The peristaltic-stimulating effects and stool softening effects of Peristim were very dependable. Peristim was shown to counteract the peristaltic-inhibiting effects of chlorisondamine and abdominal surgery under ether anesthesia. Large amounts of atropine were incapable of significantly altering the fecal-hydrating effects of Peristim in mice. It appears that there are at least two components in the total action of Peristim: (*a*) a reflex independent peristaltic-stimulating effect and, (*b*) a fecal-hydrating action which proceeds in spite of ganglionic with its effectiveness. In such cases the addition of a drug with the properties of Peristim would appear to be indicated. The usefulness of danthron as an additive agent would seem limited since it lacks the peristaltic-stimulating effects of Peristim and, like Colace, its fecal-hydrating effects are inhibited strongly by a ganglionic blocking agent.

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Investigation of Drug Release from Solids II.*

Theoretical and Experimental Study of Influences of Bases and Buffers on Rates of Dissolution of Acidic Solids

By W. I. HIGUCHI, EUGENE L. PARROTT,† DALE E. WURSTER, and T. HIGUCHI

A theory is presented which describes the rate of dissolution of solids in basic and buffered media. The formulation is based on the Nernst-Brünner film model assuming a solution diffusion controlled process. Useful expressions are obtained which give relative dissolution rates as functions of the primary variables. Results of calculations predict that for a solid weak acid dissolving in an aqueous basic solution, the rates of dissolution become essentially independent of the strength of the base above certain values. In the region where it is independent of the base strength the rate is a linear function of the product of the base concentration and the base diffusion coefficient. The theoretical conclusions are satisfactorily substantiated by experiments on the rates of dissolution of benzoic acid in basic media of varying strength and diffusivities. The following bases were used as additives: sodium hydroxide, sodium bicarbonate, sodium tetraborate, disodium phosphate, sodium acetate, and ethanolamine. Studies were also carried out with added swamping electrolyte. It was found that the rates of dissolution in varying concentrations of sodium chloride in neutral solutions can be largely explained by the salting out effects of the electrolyte.

IT IS OF IMPORTANCE both to the manufacturer and the administrator of pharmaceutical dosage forms to clearly understand the fundamental factors which influence the rate of dissolution of drugs. The kinetics problem of a substance dissolving in a nonreacting medium is relatively well understood (1–6). As physiological conditions are, more often than not, somewhat removed from neutrality, it is highly desirable to establish the primary effects which basicity and acidity induce on the dissolution rates of an acidic or basic substance. Furthermore, it is desirable to examine the influence of a neutral salt upon the dissolution rates in both neutral and reactive media.

King and Brodie (7) and Hixon and Baum (8) have reported results on the dissolution rates of benzoic acid in dilute aqueous sodium hydroxide and potassium hydroxide. They have satisfactorily explained their data on the basis of the Nernst-Brünner two film model of diffusion controlled kinetics. Their theory can be described as shown in Fig. 1. It is assumed that there exists an effective liquid film between the surface of the benzoic acid solid and the stirred bulk solution. At the solid-film interface the film is saturated with benzoic acid. The diffusion of benzoic acid through this film of thickness $x + y$ is solely responsible for the rate of dissolution. Linear concentration gradients of all species as shown in Fig. 1 are assumed to exist in this film. The rate of dissolution then is proportional to the slope of the (HA) line.

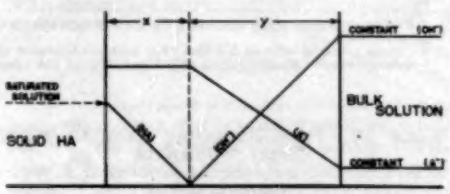

Fig. 1.—Concentration profiles during the dissolution of benzoic acid in aqueous sodium or potassium hydroxide solutions. The film thickness is $X + Y$.

It is clear that this picture neglects the effects of the base strength of the medium and the diffusivities of the reaction products. Moreover, it assumes that all of the reaction occurs at the plane where the reactants "meet," disregarding the possibility of neutralization elsewhere. Although such a model explains well systems involving a reaction equilibrium which lies very far to the right, it is expected to fail for the general case. It is one of the intents of this research to provide a general theory, substantiated by experiments, which includes the influence of the equilibrium constants among the factors considered.

The studies reported here are limited to the kinetics of the process: Weak Acid (solid) → Solution. The general conclusions however may be extended and applied to any problem in which the process is diffusion controlled in the solution phase.

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The paper is based on a dissertation submitted by Eugene L. Parrott to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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THEORY

The formulation is based on the Nernst-Brünner model (9) of a diffusion controlled process in the solution phase. It is assumed that the effective liquid film concept of representing mass transfer is applicable. The thickness of this film is assumed to be independent of the diffusivities at constant stirring rates, viscosity, and geometry of the system, and the diffusion coefficients are assumed to be independent of concentration. Furthermore, all chemical equilibria are assumed to be rapid compared to diffusion.

For the problem of the rate of dissolution of a solid weak acid, HA, in a solution of an ionic base B^- the important chemical equilibrium is

$$HA + B^- \underset{}{\overset{K_e}{\rightleftharpoons}} A^- + HB$$

where as usual

$$K_e = \frac{K_{HA}}{K_{HB}} = \frac{(A^-)(HB)}{(HA)(B^-)} \frac{\gamma_{A^-}\gamma_{HB}}{\gamma_{HA}\gamma_{B^-}}$$

is the thermodynamic equilibrium constant. (S) is the concentration of species S and γ_S is the activity coefficient of S. The function K

$$K = K_e \frac{\gamma_{HA}\gamma_{B^-}}{\gamma_{A^-}\gamma_{HB}} \frac{(A^-)(HA)}{(HA)(B^-)} \qquad \text{(Eq. 1)}$$

then is the concentration equilibrium constant.

Fig. 2 describes qualitatively the steady state situation.

At $X = O$, $(HA) = (HA)_o$, $(HB) = (HB)_o$, $(A^-) = (A^-)_o$, and $(B^-) = (B^-)_o$. (Eq. 2A)

At $X = h$, $(HA) = (HA)_h$, $(HB) = (HB)_h$, $(A^-) = (A^-)_h$, and $(B^-) = (B^-)_h$. (Eq. 2B)

For the steady state case of the one dimensional problem, the application of Fick's law (10) leads to the following set of equations:

$$\frac{d(HA)}{dt} = D_{HA}\frac{d^2(HA)}{dx^2} + \phi_1 = 0$$

$$\frac{d(HB)}{dt} = D_{HB}\frac{d^2(HB)}{dx^2} + \phi_2 = 0$$

$$\frac{d(A^-)}{dt} = D_{A^-}\frac{d^2(A^-)}{dx^2} + \phi_3 = 0$$

$$\frac{d(B^-)}{dt} = D_{B^-}\frac{d^2(B^-)}{dx^2} + \phi_4 = 0$$

D_S is the diffusion coefficient of species S in the system. The ϕ's are the source or sink functions. For proper material balance

$$D_{HA}\frac{d^2(HA)}{dx^2} = -D_{HB}d^2\frac{(HB)}{dx^2} = -D_A - \frac{d^2(A^-)}{dx^2} = D_B - \frac{d^2(B^-)}{dx^2} \qquad \text{(Eq. 3)}$$

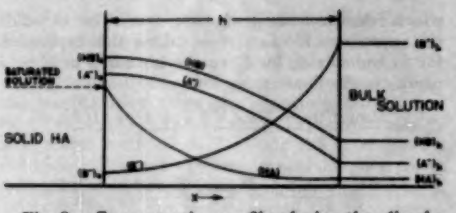

Fig. 2.—Concentration profiles during the dissolution of a weak acid, HA, in aqueous ionic base. The film thickness is h and B^- is the base ion.

Hence $\phi_1 = \phi_2 = \phi_3 = \phi_4 = O$. Equation 3 means that for a given amount of reaction of HA and B^- in any volume element, equivalent amounts of HB and A^- are produced. ϕ then is the rate of neutralization per unit volume.

The solutions to Eq. 3 obtained by integration are

$$D_{HA}\frac{d(HA)}{dx} + C_1 = -D_{HB}\frac{d(HB)}{dx} + C_2 = -D_{A^-}\frac{d(A^-)}{dx} + C_3 = D_{B^-}\frac{d(B^-)}{dx} \qquad \text{(Eq. 4)}$$

where the C's are the integration constants. The boundary conditions at $x = h$ are by material balance.

$$-D_{B^-}\frac{d(B^-)}{dx} = D_{A^-}\frac{d(A^-)}{dx} = D_{HB}\frac{d(HB)}{dx} \qquad \text{(Eq. 5)}$$

Therefore,

$$C_2 = C_3 = O$$

Also by material balance it is evident that

$$C_1 = \left(D_{B^-}\frac{d(B^-)}{dx}\right)_{x=h} - \left(D_{HA}\frac{d(HA)}{dx}\right)_{x=h} = \left(D_{HA}\frac{d(HA)}{dx}\right)_{x=o} \qquad \text{(Eq. 6)}$$

Therefore, C_1 is the rate of dissolution of HA per unit area of the film.

Now the integration of Eq. 4 with $C_2 = C_3 = O$ yields

$$D_{B^-}(B^-) = -D_{A^-}(A^-) + L_1 \qquad \text{(Eq. 7)}$$

$$D_{B^-}(B^-) = -D_{HB}(HB) + L_2 \qquad \text{(Eq. 8)}$$

$$D_{B^-}(B^-) = D_{HA}(HA) + C_1X + L_3 \qquad \text{(Eq. 9)}$$

where L_1, L_2, and L_3 are integration constants.

By appiying the boundary conditions Eq. 2b L_1 and L_2 are evaluated:

$$L_1 = D_{B^-}(B^-)_h + D_{A^-}(A^-)_h$$

and

$$L_2 = D_{B^-}(B^-)_h + D_{HB}(HB)_h$$

Combining Eq. 7, Eq. 8, and Eq. 1 and solving for $D_{B^-}(B^-)$ one obtains

$$-D_{B^-}(B^-) = \frac{D_{B^-}(L_1 + L_2) + D_{HB}D_{A^-}K(HA) \pm [\{D_{B^-}(L_1 + L_2) + D_{HB}D_{A^-}K(HA)\}^2 - 4L_1L_2D_{B^-}{}^2]^{1/2}}{2D_{B^-}}$$

which when combined with Eq. 9 at $X = O$ yields the expression for L_2. Now taking this expression for L_2 and solving for C_1 employing Eq. 9 at $X = h$ results in the final expression

$$C_1 = \frac{D_{B^-}(B^-)_h - D_{HA}(HA)_h + D_{HA}(HA)_o}{h}$$

$$- \frac{D_{B^-}(L_1 + L_2) + D_{HB}D_{A^-}K(HA)_o \pm \{[D_{B^-}(L_1 + L_2) + D_{HB}D_{A^-}K(HA)_o]^2 - 4L_1L_2D_{B^-}{}^2\}^{1/2}}{2D_{B^-}h} \quad \text{(Eq. 10)}$$

In a similar manner the problem may be solved for the analogous situation involving the nonionic equilibrium

$$HA + B \overset{K_o}{\rightleftharpoons} HAB$$

where similarly

$$K_o = \frac{(HAB)\gamma_{HAB}}{(HA)(B)\gamma_{HA}\gamma_B} = \frac{K\gamma_{HAB}}{\gamma_{HA}\gamma_B} \quad \text{(Eq. 11)}$$

The rate of dissolution per unit area of a solid HA in a solution of the base B may be shown to be

$$C_1 = \frac{D_{HA}(HA)_o - D_{HA}(HA)_h}{h} + \frac{D_BW}{h}$$

$$\left(\frac{1}{(HA)_oD_{HAB}K + D_B} - \frac{1}{(HA)_hD_{HAB}K + D_B}\right) \quad \text{(Eq. 12)}$$

where W is a constant

$$W = -D_{HAB}(HAB)_h - D_B(B)_h$$

and K is defined by Eq. 11.

When the base may react with two or more acid molecules or when more than one base is present, the problem becomes somewhat more algebraically unwieldy but the general treatment is essentially the same as that for the simpler cases. The appendix discusses the problem of HA (solid) dissolving in the presence of a diacidic base.

It can easily be shown that Eq. 10 and 12 reduce to their expected expressions in the limits:

$$K \to O$$

and

$$K \to \infty$$

Where K is very small both Eq. 10 and 12 is approximated by

$$C_1 = \frac{D_{HA}(HA)_o - D_{HA}(HA)_h}{h} \quad \text{(Eq. 13)}$$

Where K is very large Eq. 10 and 12 reduce to

$$C_1 = \frac{D_{HA}(HA)_o - D_{HA}(HA)_h + D_{B^-}(B^-)_h}{h} \quad \text{(Eq. 14)}$$

and

$$C_1 = \frac{D_{HA}(HA)_o - D_{HA}(HA)_h + D_B(B)_h}{h} \quad \text{(Eq. 15)}$$

respectively. Equations 14 and 15 predict linear concentration gradients in the film for both acid and the base. These would form the basis of the theory applied by King and Brodie (7) and Hixon and Baum (8) to their data.

Let us now ascertain the K values which would permit the use of Eq. 13 or 14 and 15 as sufficient approximations say to about 1% of Eq. 10 and 12. Since only approximate magnitudes are desired, all diffusion coefficients are assumed to be equal.

Also taking $(A^-)_h = (HB)_h = (HA)_h = O$, eq. 10 reduces to

$$C_1 = \frac{D(HA)_o}{h} - \frac{DK(HA)_o^2}{2h} + \frac{DK(HA)_o}{2h}\left(1 + \frac{4(B^-)_h}{K(HA)_o}\right)^{1/2} \quad \text{(Eq. 16)}$$

Expansion of the square root term to three terms and neglecting the other gives

$$C_1 = \frac{D(HA)_o}{h} + \frac{D(B^-)_h}{h} - \frac{2D(B^-)_h{}^2}{Kh(HA)_o} \quad \text{(Eq. 17)}$$

For Eq. 14 to approximate Eq. 10 to about 1% it is seen that K must be:

$$\begin{aligned} &K > 10^3, \text{ if } (HA)_o = (B^-)_h \\ &K > 2 \times 10^3, \text{ if } 10(HA)_o = (B^-)_h \\ &K > 2 \times 10^4, \text{ if } 100\,(HA)_o = (B^-)_h \end{aligned} \quad \text{(Eq. 18)}$$

It is found with the aid of eq. 16 that for Eq. 13 to approximate Eq. 10 to about 1% K must be:

$$\begin{aligned} &K < 10^{-4} \text{ if } (HA)_o = (B^-)_h \\ &K < 10^{-5} \text{ if } 10(HA)_o = (B^-)_h \\ &K < 10^{-6} \text{ if } 100(HA)_o = (B^-)_h \end{aligned} \quad \text{(Eq. 19)}$$

Let us now examine eq. 12 and decide how large K must be in this case for Eq. 15 to approximate Eq. 12. Assume again that $D_{HA} = D_B = D_{HAB} = D$ and that $(HAB)_h = (HA)_h = O$. It is seen from eq. 12 that K must be:

$$K > \frac{10^3}{(HA)_o} \quad \text{(Eq. 20)}$$

in order that the approximation be good to 1%. Also for the nonionic base K must be:

$$\begin{aligned} &K < 1 \text{ for } (B)_h = (HA)_o \\ &K < 10^{-1} \text{ for } (B)_h = 10(HA)_o \\ &K < 10^{-2} \text{ for } (B)_h = 100(HA)_o \end{aligned} \quad \text{(Eq. 21)}$$

in order that the base not influence the dissolution rate to more than about 1%.

As a numerical example, ionic bases having a K_B greater than about 10^{-7} will influence the rate of dissolution of benzoic acid ($K_A = 6 \times 10^{-5}$) essentially independently of their strengths. Nonionic bases having K_B greater than about 10^{-6} will also act independently of their strengths on benzoic acid dissolution rates. The above calculations refer to nonoacidic bases, of course.

EXPERIMENTAL

In order to test the theoretical conclusions of the preceding section, experiments were carried out on the rates of dissolution of benzoic acid in the presence of various alkaline additives with and without added inert electrolyte. The choice of the alka-

line substances were based on several factors: desirability of wide ranges of base strengths, types, and diffusivities. The purpose of the added neutral electrolyte was to determine the nature and the magnitudes of the salt effects. It was expected that the electrical effects of the electrolyte would modify the diffusion rates of the ionic species (11).

Apparatus and Procedure.—The apparatus and method have already been described (3). The rates at 25° were determined from both the radius and weight measurements. Two liters of various concentrations of sodium hydroxide, disodium phosphate, sodium bicarbonate, sodium acetate, sodium tetraborate, and ethanolamine were used as solvents with and without 0.75 *M* sodium chloride as an additive. Experiments were also carried out in pure sodium chloride solutions.

RESULTS AND DISCUSSION

The results are summarized in Tables I, II, and III and in Figs. 3 to 9.

TABLE I.—RATE OF DISSOLUTION OF BENZOIC ACID IN SODIUM CHLORIDE SOLUTIONS

Rate, Gm. cm.$^{-2}$ Hr.$^{-1}$	Conc. NaCl, M/L	Solubility, M/L
0.0426	0.00	0.0285
0.0414	0.154	0.0281
0.030	0.75	0.0203
0.0274	1.008	0.0188
0.0218	1.545	0.0155
0.0102	3.09	0.0082
0.0044	4.635	0.0042

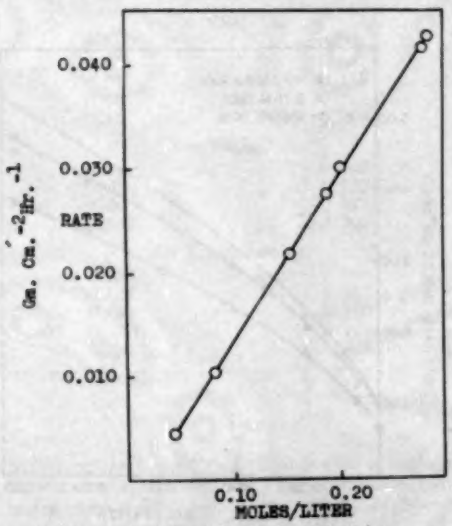

Fig. 3.—A plot of the dissolution rate *vs.* the solubility of benzoic acid in NaCl solutions of various concentration. (See Table I).

Rates in Pure Sodium Chloride Solutions.—For the discussion and later calculations, the value of h, the film thickness, is now determined. The dissolution rate of benzoic acid in pure water under the conditions of these experiments is 0.0426 Gm. cm.$^{-2}$ hr.$^{-1}$ (3). The solubility of benzoic acid in pure water is 0.0285 *M*. Taking the value for the diffusion coefficient of benzoic acid in pure water measured by King and Brodie (7), *viz.* $D_{HA} = 1.11 \times 10^{-5}$ cm.^{2} sec.$^{-1}$, one obtains, using Eq. 13,

$$h = 3.28 \times 10^{-3} \text{ cm.}$$

From the slope of the apparently linear plot of Fig. 3 and Eq. 13 the diffusion coefficient of benzoic

TABLE II.—RATE OF DISSOLUTION OF BENZOIC ACID TABLETS IN VARIOUS CONCENTRATIONS OF SOME ALKALINE SUBSTANCES

Concentration, Moles/Liter	Rate, Radius Method	Rate, Weight Method
	For NaOH	
0.0125	0.070	0.072
0.0312	0.139	0.135
0.0625	0.250	0.243
0.125	0.455	0.444
0.222	0.732	0.732
0.250		0.871
	For CH_3COONa	
0.013	0.057	0.059
0.015	0.055	0.056
0.050	0.090	0.092
0.052	0.100	0.096
0.10	0.127	0.130
0.15	0.160	0.158
0.20	0.177	0.183
0.25	0.206	0.204
0.25	0.195	0.204
0.33	0.213	0.227
	For Na_2HPO_4	
0.0113	0.058	0.060
0.05	0.107	0.111
0.10	0.167	0.169
0.113	0.188	0.190
0.20	0.273	0.290
0.25	0.344	0.363
	For $Na_2B_4O_7$	
0.01	0.069	0.070
0.05	0.179	0.184
0.10	0.315	0.339
0.12	0.336	0.396
0.15	0.443	0.474
	For $NaHCO_3$	
0.025	0.060	
0.05	0.077	
0.10	0.100	
0.15	0.107	
0.20	0.110	
0.20	0.123	
	For Ethanolamine	
0.02		0.070
0.032		0.098
0.05		0.124
0.089		0.184
0.158		0.291
0.206		0.384
0.275		0.471

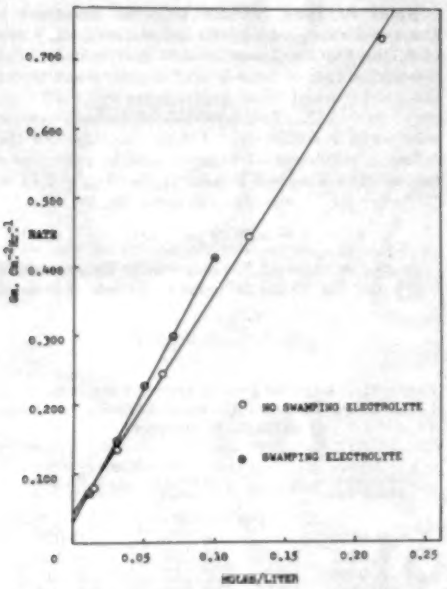

Fig. 4.—The rate of dissolution of benzoic acid in sodium hydroxide solutions.

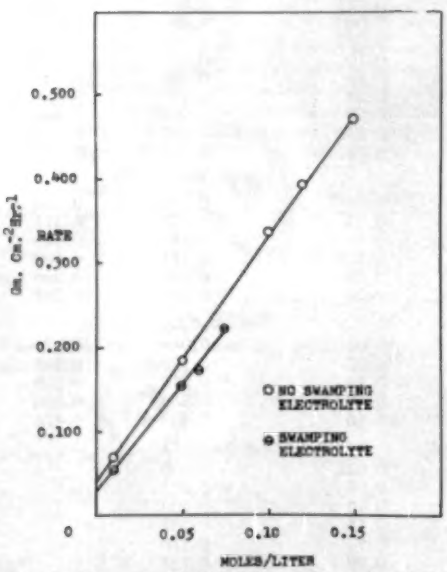

Fig. 5.—The rate of dissolution of benzoic acid in sodium tetraborate solutions.

acid in sodium chloride solution is obtained, *viz.* $D_{HA} = 1.16 \times 10^{-5}$ cm.^{2} sec.$^{-1}$. The linearity of the plot and the relatively close agreement of the diffusion coefficients over a wide range of sodium chloride concentrations may be ascribed to: the validity of Eq. 13 and the diffusion coefficient of benzoic acid being relatively constant over the range of 0 to 4 *M* sodium chloride. Since the decrease in solubility is due to the salting-out effect of the elec-

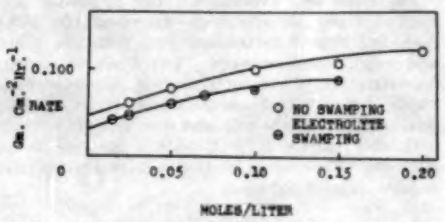

Fig. 6.—The rate of dissolution of benzoic acid in sodium bicarbonate solutions.

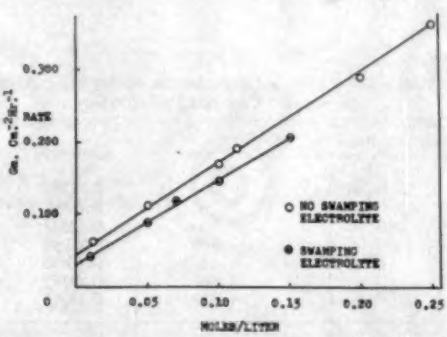

Fig. 7.—The rate of dissolution of benzoic acid in disodium phosphate solutions.

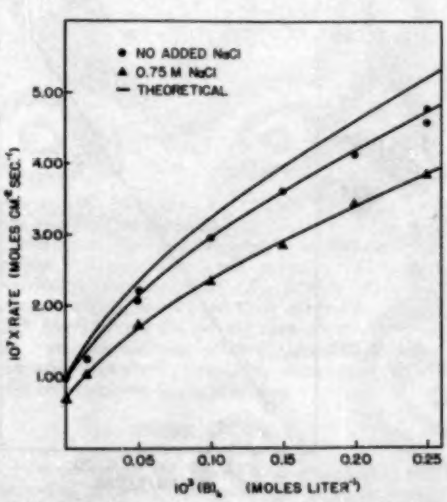

Fig. 8.—The dissolution rate of benzoic acid in aqueous sodium acetate. $(B)_A$ is the sodium acetate concentration.

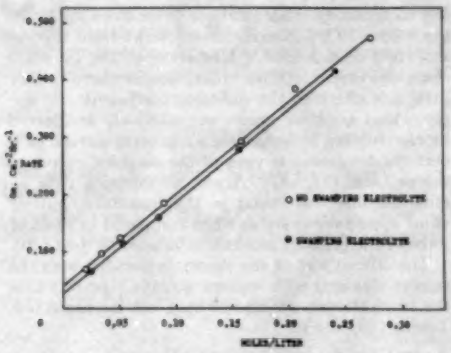

Fig. 9.—The rate of dissolution of benzoic acid in ethanolamine solutions.

TABLE III.—RATE AND CONCENTRATION OF ALKALINE IONIC SUBSTANCE IN THE PRESENCE OF A SWAMPING ELECTROLYTE (0.75 *M* NaCl)

Concentration, Moles/Liter	Rate
For NaOH	
0.015	0.078
0.03	0.147
0.05	0.227
0.07	0.298
0.10	0.413
For CH_3COONa	
0.015	0.045
0.05	0.077
0.10	0.103
0.15	0.124
0.20	0.151
0.25	0.168
For Na_2HPO_4	
0.01	0.041
0.05	0.087
0.07	0.118
0.10	0.143
0.15	0.204
For $Na_2B_4O_7$	
0.01	0.055
0.05	0.153
0.06	0.173
0.075	0.222
For Ethanolamine	
0.024	0.066
0.052	0.117
0.085	0.160
0.156	0.278
0.242	0.418

trolyte, it can be said that the influence of sodium chloride on the dissolution rate of benzoic acid under these conditions is almost wholly explained by this salting out effect.

Linear and Nonlinear Increase of Rates with Base Concentrations.—Except for Figs. 6 and 8 which are plots of the rates in sodium bicarbonate and sodium acetate respectively, all plots are essentially straight lines. For these the linear expressions Eq. 14 or 15 are applicable, i. e., their respective K values are sufficiently great so that the approximate expression suffices. The K values are: 6×10^3 for sodium hydroxide, 1×10^3 for boric acid, 1×10^3 for disodium phosphate (reaction with one hydrogen equivalent), and 2×10^3 for ethanolamine. All meet conditions Eq. 18 or 20. For these substances the rate of dissolution of benzoic is uninfluenced by their base strengths.

A calculation will show that the reaction equilibrium

$$HA + H_2PO_4^- \rightleftharpoons H_3PO_4 + A^-$$

where HA = benzoic acid, is always far to the left throughout the film. As the benzoate ion concentration is relatively large, the ratio $H_3PO_4/H_2PO_4^-$ is nowhere greater than about 1%. Hence for benzoic acid dissolution, under the conditions of these experiments disodium phosphate will act monoacidic. The general case of a weak acid solid dissolving in the presence of a diacid base should be treated according to the method discussed in the appendix.

A tetraborate ion is interpreted as two borate ions (12). Although at the higher base concentrations there may be appreciable tetraborate ions in the stirred solution, probably most of the diffusing species and certainly all of the reacting species are borate ions.

The plots of the rates of dissolution of benzoic acid in sodium bicarbonate solution both with and without a swamping electrolyte indicate (Fig. 6) marked deviations from linearity. As the K for this case is about 200, linearity to better than 10% was predicted by Eq. 17. The deviations are ascribed to carbon dioxide gas pockets which were visually observed at the solid-solution interface, generating an added resistance which varied with the concentration of the bicarbonate. Calculations in the next section confirm this conclusion. A mathematical treatment of a problem of this type is seriously complicated by the nonequilibrium conditions existing at the interface. The surface properties of the solid will determine to a large extent the ease of liberation of the gas, and hence the rate of dissolution.

The nonlinear behavior in the presence of sodium acetate was expected in accordance with the theory. The K value of about 3.7 is too small for Eq. 14 to apply and yet too large for the alkaline effects to be neglected. Hence only the complete expression (10) will apply. The test of (10) for this case is carried out below.

Some deviations from linearity, though generally of much smaller magnitude than those observed with sodium carbonate and sodium acetate, may be expected as consequences of the assumptions underlying the theory even for those systems where $K > 10^3$. The diffusion coefficients may vary with the concentration and hence with distance across the film. They may also vary with the different ionic and nonionic species present at various concentrations across the film. The film thickness has been noted (13, 14, 8) to be not entirely independent of the diffusivities. The salting-in effect of the benzoate ion (15) will to some ex-

tent affect (*HA*). Nevertheless, all of these effects influence to only a small extent the linearity of a system where $K > 10^3$ as demonstrated by the plots.

Quantitative test of theory and the effects of an added electrolyte.—Employing Eq. 14 or 15 where applicable the diffusion coefficients may be calculated from the experimental results and compared with values in the literature or with values estimated from conductance data. Unfortunately the literature data are scanty, and where available, usually pertain to conditions somewhat removed from the situation of the experiments here. From the slopes of the straight lines in Figs. 4, 5, 6, 7, and 9 the diffusion coefficients are calculated as

$$D_{B^-} = \text{slope}/h = \text{slope}/3.3 \times 10^{-2}\text{cm.}$$

Table IV, columns two and three, give these calculated values. For sodium hydroxide the agreement with the limiting value and King and Brodie's value is good. In this case the effect of 0.75 *M* sodium chloride is to increase the diffusion coefficient of the hydroxide ion by about 12%.

low bicarbonate concentrations is far from the limiting value. This disagreement follows from the observation of gas bubble formation at the interface discussed earlier. With ethanolamine there is very little salt effect on the diffusion coefficient. It appears that nonionic agents are relatively unaffected by electrolytes, although such a general statement is perhaps dangerous in view of the complex nature of the problem (17, 18). Again, no diffusion data on ethanolamine was found in the literature, but its value appears reasonable when compared to those of some of the primary alcohols tabulated by Jost (10).

The crucial test of the theory is possible with the results obtained with sodium acetate. In this case the bracket term of Eq. 10 may not be neglected. Taking (10) and for

$$D_{A^-}(A^-)_h = D_{HB}(HB)_h = D_{HA}(HA)_h = 0$$

$$C_1 = \frac{D_{HA}(HA)_o}{h} - \frac{D_{HB}D_{A^-}K(HA)_o}{2D_{B^-}h} + \frac{D_{HB}D_{A^-}K(HA)_o}{2D_{B^-}h}\left(1 + \frac{4D_{B^-}(B^-)_h}{D_{HB}D_{A^-}K(HA)_o}\right)^{1/2}$$

TABLE IV.—EXPERIMENTAL DIFFUSION COEFFICIENTS FROM RATES OF DISSOLUTION OF BENZOIC ACID

Substance	D_{B^-} or D_B, No Added Electrolyte	D_{B^-} or D_B, With 0.75 *M* NaCl	D_{B^-} or D_B Literature
Sodium hydroxide	2.42×10^{-5}	2.92×10^{-5}	2.42×10^{-5}[a] $2.7\text{–}2.9 \times 10^{-5}$[b]
Sodium borate (Sodium tetraborate)	1.03×10^{-5}	0.93×10^{-5}	
Disodium phosphate	0.97×10^{-5}	0.87×10^{-5}	1.06×10^{-5}[a]
Ethanolamine	1.22×10^{-5}	1.20×10^{-5}	
Sodium bicarbonate	5.0×10^{-5}[c]	6.4×10^{-5}[c]	1.25×10^{-5}[a]

Units of cm.^{2} sec.$^{-1}$.
[a] Using conductance data tabulated in references 11 and 16 and using Nernst's and Haskell's limiting equations (reference 11) for infinite dilution.
[b] King's recommended value, reference 7.
[c] Initial slopes used.

This increase is primarily due to the suppression of the diffusion potential (11) created by the sodium ion, overwhelming the usual electrolyte depression of the diffusion coefficient of ionic substances as observed in the cases of disodium phosphate and sodium borate. This diffusion potential arises when the diffusing ions do not all have about the same mobility. It is particularly noticeable in systems involving hydrogen or hydroxide ions (11) and is probably of secondary importance in the cases of disodium phosphate and sodium borate. In the latter the diffusion coefficients decrease with added electrolyte as is generally expected (17, 18). With disodium phosphate the agreement with limiting value is satisfactory. Here the limiting value is expected to be greater than the observed value in column 1, and by about the amount observed, *viz.* ~10% (11). Unfortunately no conductance data could be found for sodium borate and hence a check was not possible. However, the observed value of 1.03×10^{-5} appears to be reasonable when compared with 0.97×10^{-5} of the disodium phosphate, or with the limiting value of 1.25×10^{-5} for sodium bicarbonate. In the case of sodium bicarbonate even the diffusion coefficient obtained at

The following values are employed: $K = 3.67$, $(HA)_o = 0.0285$ *M*, $h = 3.3 \times 10^{-3}$ cm., $D_{HB} = 1.28 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ (extrapolated from values at 0.1 *M* and 18° and below in reference 19), $D_{B^-} = 1.19 \times 10^{-5}$ (references 11, 16, limiting value), $D_{HA} = 1.11 \times 10^{-5}$, $D_{A^-} = 1.04 \times 10^{-5}$ (reference 11, 16, limiting value).

Then,

$$C_1 = 1.78 \times 10^{-7}\,(1 + 4.13 \times 10^4\,[B^-]_h)^{1/2} - 0.81 \times 10^{-7} \text{ moles cm.}^{-2} \text{ sec.}^{-1}$$

The plot of this equation is given in Fig. 8. The agreement must be considered satisfactory recalling that only approximate values for the diffusion coefficients were used. In choosing $K = 3.67$ it was assumed that the thermodynamic salt effects cancel in the reaction, and therefore the activity coefficients cancel. It is notable that the experimental curve without the swamping electrolyte falls between the theoretical curve and the experimental curve with the added electrolyte. This salt effect trend was also observed with disodium phosphate and sodium tetraborate, but reversed in the case of sodium hydroxide for reasons already given. It appears safe to state that, except for

bases involving abnormally rapid or slow ions, sodium chloride at 0.75 M will decrease the base controlled rates of dissolution of benzoic acid by about 10 to 20% aside from that due to the salting out effects.

APPENDIX

Dissolution of Acidic Solids in Presence of Diacidic Base

For the case of HA (solid) dissolving in the presence of a diacidic base, $B^=$, the important equilibria are

$$HA + B^= \underset{\rightleftarrows}{K_1} HB^- + A^-, \; K_1 = \frac{(HB^-)(A^-)}{(HA)(B^=)}$$

and (1A)

$$HB^- + HA \underset{\rightleftarrows}{K_2} H_2B + A^-, \; K_2 = \frac{(H_2B)(A^-)}{(HB^-)(HA)}$$

where K_1 and K_2 are concentration equilibrium constants. The appropriate differential equations to be solved are

$$D_{HA}\frac{d^2(HA)}{dx^2} = D_{B^=}\frac{d^2(B^=)}{dx^2} - D_{HB^-}\frac{d^2(H_2B)}{dx^2} = -D_{A^-}\frac{d^2(A^-)}{dx^2}$$

and (2A)

$$D_{B^=}\frac{d^2(B^=)}{dx^2} = -D_{HB^-}\frac{d^2(HB^-)}{dx^2} - D_{H_2B}\frac{d^2(H_2B)}{dx^2}$$

The solutions to (2A) employing (1A) are

$$D_{B^=}(B^=) = -D_{HB^-}\frac{(HA)(B^=)K_1}{(A^-)} - D_{H_2B}(H_2B) + L_1,$$

$$D_{B^=}(B^=) = -D_{HB^-}\frac{(H_2B)(A^-)}{K_2(HA)} - D_{H_2B}(H_2B) + L_1,$$

$$D_{B^=}(B^=) = D_{H_2B}(H_2B) - D_{A^-}(A^-) + L_2,$$

and

$$D_{B^=}(B^=) + C_1X = D_{HA}(HA) + D_{H_2B}(H_2B) + L_3.$$

L_1, L_2, and L_3, and C_1 are constants, where C_1 is the rate of dissolution of HA per unit area as before. L_1 and L_2 may be taken equal to $D_{B^=}(B^=)_h$ if $(HB^-)_h = (H_2B)_h = 0$. The problem remaining is to evaluate L_3 and then C_1 algebraically. The explicit solution would involve a quartic equation. Therefore the method of successive approximations would be more convenient. The final set of two equations to be solved are

$$(|D_{B^=}(B^=) - L_1|^2 - K_1D_{HB^-}(HA)(B^=)D_{A^-})^{1/2} = -\frac{1}{2D_{HB^-}}(L_2D_{HB^-} - D_{HB^-}D_{B^=}(B^=) + K_2D_{A^-}D_{H_2B}(HA)) + \frac{1}{2D_{HB^-}}\{(L_2D_{HB^-} - D_{HB^-}D_{B^=}(B^=) + K_2D_{A^-}D_{H_2B}(HA)^2 + 4L_1D_{HB^-}D_{H_2B}D_{A^-}(HA)K_2 - 4K_2D_{A^-}D_{B^=}D_{HB^-}D_{H_2B}(HA)(B^=)\}^{1/2}$$

and

$$(B^=)\{2D_{B^=}(C_1X - D_{HA}(HA) - L_2 + 2D_{B^=}L_1 + K_1D_{HB^-}D_{A^-}(HA)| = L_1^2 - (C_1X - D_{HA}(HA) - L_3)^2$$

Now these equations may be solved at $x = 0$ and $x = h$ and C_1 evaluated as a function of $(B^=)_h$, $(HA)_0$, K_1, K_2, and the diffusion coefficients.

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Book Notices

A Survey of State Pharmacy Laws. Compiled by JOHN M. SEUS for the National Drug Trade Conference. The American Pharmaceutical Association, Washington, D. C., 1957. vi + 112 pp. 16.5 x 25 cm. Price $3.

This compilation was prepared primarily to correlate the many different versions of those sections of the various State pharmacy laws pertaining to restriction of the sale of nonprescription drugs, so that the National Drug Trade Conference could use the information in again attempting to prepare a generally satisfactory "model" State pharmacy act. The text includes three general chapters on: Provision of State statutes as to sale of nonprescription drugs; Powers granted to Boards of Pharmacy, Power exercised by Boards of Pharmacy; and Decisions of the courts as to definitions of the term, "patent medicines," "proprietary medicines," "household and domestic remedies," and classification of articles according to restriction of sale, and other related matters. The 63 appendixes include various tables classifying States as to the provisions of their respective laws regulating the sale of drugs by general merchants, and excerpts of specific references to the laws in the 48 States, the District of Columbia, and the Territories of Alaska and Hawaii.

This valuable compilation should be a useful reference for anyone interested in the restrictive sale phase of pharmacy laws. No general index is given but the comprehensive Table of Contents serves the purpose of a detailed guide to the text.

Bacterial Fermentations. CIBA Lectures in Microbial Biochemistry. By H. A. BARKER. John Wiley & Sons, Inc., New York, 1957. vii + 95 pp. 12.5 x 18.5 cm. Price $3.

This small book presents the three lectures, by the author, on those aspects of bacterial fermentation which he has investigated or which have been of particular interest to him. The subjects covered are: Biological formation of methane, The chemistry of butyric acid-butanol fermentations, and Fermentations of nitrogenous compounds. The main emphasis in each lecture is placed upon the chemistry of the energy-yielding processes. References are given after each chapter and index is appended.

Dermatologic Formulary. From the New York Skin and Cancer Unit, Service of Dermatology. 2nd ed. By FRANCES PASCHER. Paul B. Hoeber, Inc., Medical Book Department of Harper & Bros., New York, 1957. xii + 172 pp. 12.5 x 19 cm. Price $4.

This book represents a hospital formulary committee's compilation of "The most tried and useful dermatologic prescriptions, together with the briefest and simplest explanations of their uses, indications, and contraindications." The sections of the text are introduced with brief discussions relating to: Topical remedies, Systemic therapy, Articles for clinic use, and Therapeutic aids. A sufficient number of medicaments are included to circumvent allergic and idiosyncratic reactions and to allow for different patient responses to particular drugs. The abbreviated monographs include descriptions and constituents of drugs and dosage forms.

The book is intended for the dermatologist and does not include procedures for the preparation of dosage forms. An index, with references to prescription numbers and not page numbers, is appended. The book will undoubtedly serve as a model for formularies for other hospital dermatology clinics.

Dictionary of Scientific Terms. By I. F. HENDERSON and W. D. HENDERSON. 6th ed. Revised and enlarged by JOHN H. KENNETH. D. Van Nostrand Co., Inc., New York, 1957. xvi + 532 pp. 14 x 22.5 cm. Price $12.50.

This dictionary aims to give the pronunciation, derivation, and definition of some 14,000 terms in biology, botany, zoology, anatomy, cytology, genetics, embryology, and physiology. Specific, generic, ordinal, and other systematic names of plants and animals were omitted to limit the size of the book. The method of spelling is in the main that used in Britain with American orthography indicated in cross-references or by reproducing in the original lettering terms culled from U. S. literature. The book should be very useful in the indicated fields of biological sciences.

Handbook of Chemical Data. Edited by F. W. ATACK. Reinhold Publishing Corp., New York, 1957. 629 pp. 10 x 15 cm. Price $6.75.

This pocket-size book includes tabulations of data that are most frequently consulted by chemists. Its main feature is the small size, which is achieved by judicious omission of less frequently required information which can be found in larger volumes.

Pharmaceutical Calculations. 3rd ed. By WILLIS T. BRADLEY, CARROLL B. GUSTAFSON, and MITCHELL J. STOKLOSA. Lea & Febiger, Philadelphia, 1957. 13 x 20 cm. 325 pp. Price $4.50.

This revision of a book that has been used by many teachers has been brought up to date and conforms with the usage of U. S. P. XV. It is designed to prepare the student for the use of arithmetic in applied pharmacy. This is done with the aid of many examples and problems. The book is well suited for the modern pharmacy curriculum, and should be useful to practitioners as well as students and teachers.

Organic Syntheses. Vol. 37. Edited by JAMES CASON. John Wiley & Sons, Inc., New York, 1957. vii + 109 pp. 15 x 23 cm. Price $4.

This volume presents preparative methods for twenty-nine compounds frequently required by organic chemists.

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